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Bacterial Degradation of Dibenzo-p-dioxin and Chlorinated Dibenzo-p-dioxins

Texas Univ. at Austin

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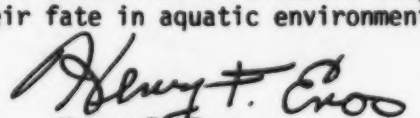
FOREWORD

The protection of our estuarine and coastal areas from damage caused by toxic organic pollutants required that regulations restricting the introduction of these compounds into the environment be formulated on a sound scientific basis. Accurate information describing dose-response relationships for organisms and ecosystems under varying conditions is required. The Environmental Research Laboratory, Gulf Breeze contirbutes to this information through research programs aimed at determining:

- the effects of toxic organic pollutants on individual species and communities of organisms;
- the effects of toxic organics on ecosystem processes and components;
- the significance of chemical carcinogens in the estuarine and marine environment.

This report sought to determine the initial oxidative pathways for dibenzodioxin and dibenzofuran compounds by bacteria in aquatic systems. Bacterial cultures were isolated and oxidized dibenzo-p-dioxin and mono-chlorinated dibenzo-p-dioxins; however, di-chlorinated dibenzo-p-dioxins, were not metabolized by either strain. The metabolite, 1,2-dihydrodibenzo-p-dioxin, was a potent inhibitor of further oxidation. These results will aid in the understanding of bacterial metabolic mechanisms of aromatic compounds and eventually may help to predict their fate in aquatic environments.



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ABSTRACT

Pseudomonas sp. N.C.I.B. 9816, strain 11, when grown on salicylate in the presence of dibenzo-*p*-dioxin, accumulated *cis*-1,2-dihydroxy-1,2-dihydrodibenzo-*p*-dioxin and 2-hydroxydibenzo-*p*-dioxin in the culture medium. Each metabolite was isolated in crystalline form and identified by a variety of conventional chemical techniques. Crude cell extracts prepared from the parental strain grown with naphthalene oxidized *cis*-1,2-dihydroxy-1,2-dihydrodibenzo-*p*-dioxin under both aerobic and anaerobic conditions to 1,2-dihydroxydibenzo-*p*-dioxin. Further degradation of this metabolite was not detected.

Whole cells of the parent strain of *Beijerinckia*, grown with succinate and biphenyl, oxidized dibenzo-*p*-dioxin and several chlorinated dioxins. The rate of oxidation of the chlorinated dibenzo-*p*-dioxins decreased with an increasing degree of chlorine substitution. A mutant strain (B8/36) of *Beijerinckia* oxidized dibenzo-*p*-dioxin to *cis*-1,2-dihydroxy-1,2-dihydrodibenzo-*p*-dioxin. The mutant organism also oxidized two monochlorinated dibenzo-*p*-dioxins to *cis*-dihydrodiols. No metabolites were detected from two dichlorinated dibenzo-*p*-dioxins. Growth of the parent strain of *Beijerinckia* on succinate was inhibited after four hours when 0.05% dibenzo-*p*-dioxin was present in the culture medium. Resting cell suspensions of the parent organism, previously grown with succinate and biphenyl, oxidized dibenzo-*p*-dioxin to a compound identified as 1,2-dihydroxydibenzo-*p*-dioxin. Further degradation of this metabolite was not detected, as the compound was found to be a potent mixed-type inhibitor of two ring-fission oxygenases present in this organism.

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BACTERIAL DEGRADATION OF DIBENZO-p-DIOXIN AND CHLORINATED DIBENZO-p-DIOXINS

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CONTENTS

Foreword iii
Abstract iv
Figures vi
Tables viii

1. Introduction 1
2. Conclusions 3
3. Recommendations 4
4. Materials and Methods 5
5. Results and Discussion 12

References 52

FIGURES

Number		Page
1	Thin-layer chromatogram of the products formed from the oxidation of dibenzo-p-dioxin by Pseudomonas sp. N.C.I.B. 9816 strain 11	16
2	Formation of compound A by Pseudomonas sp. N.C.I.B. 9816 strain 11	17
3	Infrared spectrum of compound B (Nujol)	18
4	Ultraviolet spectrum of compound B (MeOH)	19
5	Proton magnetic resonance spectrum of compound B (Acetone-d_6)	20
6	Infrared spectrum of compound A (Nujol)	22
7	Ultraviolet spectrum of compound A (MeOH)	23
8	Proton magnetic resonance spectrum of compound A (Pyridine-d_5)	24
9	Infrared spectrum of compound C (Nujol)	27
10	Ultraviolet spectrum of compound C (MeOH)	28
11	Proton magnetic resonance spectrum of compound C ($CDCl_3$)	29
12	Infrared spectrum of compound D (Nujol)	32
13	Ultraviolet spectrum of compound D (MeOH)	33
14	Proton magnetic resonance spectrum of compound D (Acetone-d_6)	34
15	Polarographic assay of 1,2-dihydroxynaphthalene oxygenase in cell-free extracts of Pseudomonas sp. N.C.I.B. 9816	36
16	Growth and accumulation of compound IA by cultures of Beijerinckia B8/36	39

Number		Page
17	Growth and accumulation of compounds IIA and IIIA by cultures of *Beijerinckia* B8/36	41
18	Acid-catalyzed dehydration of compounds IIA and IIIA	43
19	Effect of dibenzo-*p*-dioxin on the growth of *Beijerinckia* sp., wild type	45
20	Polarographic assay of ring-fission oxygenases in cell extracts of *Beijerinckia* sp.	46
21	Separation of the 2,3-dihydroxybiphenyl and catechol oxygenases by ion-exchange (DEAE) chromatography	48
22	Effect of 1,2-dihydroxydibenzo-*p*-dioxin on the ring-fission oxygenases from *Beijerinckia* sp.	51

TABLES

Number		Page
1	Summary of the preliminary screening of organisms for the ability to co-oxidize dibenzo-p-dioxin	13
2	Oxidation of substrates by washed, whole cells of *Pseudomonas* sp. N.C.I.B. 9816	14
3	Analysis of the proton magnetic resonance spectrum of compound B	21
4	Analysis of the proton magnetic resonance spectrum of compound A	25
5	Analysis of the proton magnetic resonance spectrum of compound C	30
6	Analysis of the proton magnetic resonance spectrum of compound D	35
7	Oxidation of substrates by washed, whole cells of *Beijerinckia* sp.	37
8	Physical properties of the metabolites formed from mono-chlorinated dibenzo-p-dioxins by *Beijerinckia* B8/36.	42
9	Polarographic analysis of the activities of the partially purified 2,3-dihydroxybiphenyl and catechol oxygenases	50

SECTION 1

INTRODUCTION

Heterocyclic aromatic compounds containing 2 atoms of oxygen, such as dibenzo-p-dioxin have been identified in the neutral fractions of bituminous coal (16). Interest in chlorinated dibenzo-p-dioxins arose with the identification by x-ray diffraction of 1,2,3,7,8,9-hexachlorodibenzo-p-dioxin as the "chick edema factor" which caused the deaths of millions of broiler chickens in 1957 (4). It is now known that conditions utilized in the synthesis of certain chlorinated fungicides and herbicides, such as pentachlorophenol, 2,4,5-trichlorophenol and 2,4,5-trichlorophenoxyacetic acid can result in the formation of chlorinated dibenzo-p-dioxins. The most potent isomer, 2,3,7,8-tetrachlorodibenzo-p-dioxin has been considered to be one of the most toxic low molecular weight compounds known to man, with a mean lethal dose in guinea pigs of 1.0 μg/kg of body weight (27). The high toxicity of some of these contaminants, when considered in light of the extensive worldwide use of chlorophenols in both industry and agriculture, is of environmental concern.

The potential for the degradation of chlorinated dibenzo-p-dioxins in the environment has been investigated. Reductive dechlorination has been reported by several authors (7,8). The reaction required ultraviolet light and the presence of a suitable organic hydrogen donor. Kearney et al. (18) have monitored the metabolism and persistence of ^{14}C-labelled 2,7-dichlorodibenzo-p-dioxin and 2,3,7,8-tetrachlorodibenzo-p-dioxin in experimental soil samples.

2,7-Dichlorodibenzo-p-dioxin was oxidized to $^{14}CO_2$ and to one or more polar metabolites detected in ethanol extracts of the soil samples. Attempts to identify the metabolites were unsuccessful. 2,3,7,8-Tetrachlorodibenzo-p-dioxin was oxidized to a lesser extent, as over 50% of the original samples were recovered after one year. No metabolites were detected in soils treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. Matsumura & Benezet (21) screened over 100 microbial strains previously shown to be capable of degrading persistent pesticides for the ability to degrade ^{14}C-labelled 2,3,7,8-tetrachlorodibenzo-p-dioxin. Only 5 strains were found to be capable of degrading this compound to unidentified polar metabolites.

This report describes the oxidation of dibenzo-p-dioxin and several chlorinated dibenzo-p-dioxins by a Pseudomonas species and a Beijerinckia species. Evidence is presented for the identity of metabolites prior to the enzymic fission of the aromatic nucleus. Further evidence is presented that describes the inhibition by 1,2-dihydroxydibenzo-p-dioxin of enzymes responsible for the oxidative fission of aromatic molecules.

SECTION 2

CONCLUSIONS

The results of laboratory studies on the biodegradation of dibenzo-p-dioxin and its chlorinated derivatives suggest that microorganisms capable of oxidizing these compounds are relatively rare in the environment. The unsubstituted parent compound, dibenzo-p-dioxin, was shown to inhibit the growth of a laboratory strain of Beijerinckia and this physiological effect may be due to the metabolism of dibenzo-p-dioxin to 1,2-dihydroxydibenzo-p-dioxin. The latter compound was shown to be a potent inhibitor of ring-fission dioxygenases in this organism.

Dibenzo-p-dioxin and two monochlorinated derivatives were oxidized to cis-dihydrodiols by a Beijerinckia species. Pseudomonas sp. N.C.I.B. 9816 also oxidized dibenzo-p-dioxin to a cis-dihydrodiol. These observations indicate that microorganisms capable of oxidizing this class of compounds use a dioxygenase enzyme system to incorporate molecular oxygen into the aromatic nucleus. This type of metabolism is similar to that observed in the biodegradation of aromatic hydrocarbons and related compounds. Increasing chlorine substitution on the aromatic rings of dibenzo-p-dioxin decreases the rate of biological oxidation.

SECTION 3

RECOMMEDNATIONS

It is recommended that further studies on the microbial oxidation of dibenzo-p-dioxin and its chlorinated derivatives be conducted. Preliminary observations suggest that dibenzo-p-dioxin itself inhibits the growth of bacteria and the mechanism of this inhibition should be elucidated. A broader range of microorganisms including bacteria, fungi, yeasts and alga should be examined for their ability to oxidize this class of compounds in order to determine the potential fate and effects of dioxins in the environment.

SECTION 4

MATERIALS AND METHODS

Organisms and Growth Conditions

Four strains of bacteria were examined for the ability to oxidize dibenzo-p-dioxin and chlorinated dibenzo-p-dioxins. Pseudomonas sp. N.C.I.B. was kindly provided by Professor W.C. Evans, University of North Wales, Great Britain. The organism was isolated from farm soil by elective culture, and has the ability to grow on naphthalene as the sole source of carbon (9). A spontaneous mutant, Pseudomonas sp. N.C.I.B. 9816 strain 11, was isolated from the parent organism, that co-oxidized naphthalene to cis-1,2-dihydroxy-1,2-dihydronaphthalene when grown on succinate or salicylate as the carbon source (D.T. Gibson, unpublished results).

The isolation and characterization of Beijerinckia sp. has been reported (13). The organism was isolated from a polluted stream by virtue of its ability to grow with biphenyl as a sole source of carbon. Treatment of the parent organism with N-methyl-N'-nitro-N-nitrosoguanidine led to the isolation of a mutant strain, Beijerinckia B8/36, that oxidizes several different aromatic hydrocarbons to cis-dihydrodiols (12,13,17,19).

Organisms were maintained on agar slants of a mineral salts medium (29) containing 0.2% succinate, and were stored at 4 C. Cells were transferred from a slant to 100 ml of mineral salts broth (pH 7.2) containing 0.2% succinate in a 500 ml Erlenmeyer flask. Cultures were incubated at 27 C on a rotary shaker at 150 rpm for 12 hours. A 5% (v/v) inoculum of the above

culture was used for further experiments.

Biotransformation experiments were conducted in 2 liter Erlenmeyer flasks containing 500 ml of mineral salts medium as above, with the exception that the phosphate buffering capacity was double strength (pH 7.2). Carbon sources were added to the culture medium according to the experimental design. Naphthalene, biphenyl, and dibenzo-p-dioxins were added to cultures in 10.0 ml of acetone to give a final concentration of 0.05% in the culture medium. Acetone was not a growth substrate nor did it inhibit the growth of the organisms. Flasks were incubated as described above. Growth of the cultures was monitored turbidometrically at 600 nm. When samples of the cultures were filtered through glass wool, the aromatic hydrocarbons did not interfere with the measurement of cell density.

Large-scale biotransformation experiments with Pseudomonas sp. N.C.I.B. 9816 were conducted in a 50 liter carboy containing 10 liters of medium with 0.1% salicylate and 0.05% dibenzo-p-dioxin. Cultures were incubated on a reciprocating shaker at 27 C.

Cells of Pseudomonas sp. N.C.I.B. 9816 and Beijerinckia sp. used for the preparation of cell extracts were obtained from large-scale incubations (10 liter) grown with forced aeration in a New Brunswick model M14 Microferm fermentor at 30 C for 12 hours. Air was supplied at a rate of 10 liters/min and the culture was stirred at 500 rpm. Pseudomonas sp. N.C.I.B. 9816 was grown with 0.1% naphthalene. Beijerinckia sp. was grown with 0.2% succinate and 0.05% biphenyl as an inducing substrate. In addition, an anion exchange resin (BioRad AG 1-X8: 5 g/l) was added to the latter cultures to remove acid products (19). Cultures were initially filtered through glass wool to remove solid materials, and the cells harvested with an air-driven Sharples continu-

ous-flow centrifuge. The cells were washed twice with 0.05 M KH_2PO_4 buffer, pH 7.2, and used in further studies. Unused cells were stored at -15 C until required.

Detection of Metabolites

Quantitative measurement of product accumulation in the culture medium was conducted at various time intervals. A 5.0 ml aliquot of the culture was extracted with 15.0 ml of ethyl acetate. The organic extract was dried over anhydrous Na_2SO_4 and the solvent removed *in vacuo* at 30 C. The residue was applied with 0.5 ml of acetone to a glass preparative thin-layer chromatography plate, precoated with Silica-Gel 60. The solvent system used for chromatography was chloroform/acetone (80:20). Compounds were located with the use of ultraviolet light. Regions of the plate containing suspected metabolites were removed and extracted with 5.0 ml of methanol. A sample (0.1 ml) of the methanol extract was diluted to 1.0 ml with the same solvent and the ultraviolet spectrum recorded. The reference cuvette contained methanol.

Alternatively, samples of duplicate culture extracts were examined on analytical thin-layer chromatography sheets. Following development of the chromatograms as above, compounds were located with the use of ultraviolet light and also by spraying with a 2% solution of 2,6-dichloroquinone-4-chloroimide in methanol (10; Gibbs reagent). Subsequent exposure of the chromatogram to ammonia vapors often enhanced the color intensities of the different products.

Isolation of Transformation Products

When product accumulation in a culture had reached a maximum, the culture was filtered through glass wool to remove unused solid materials. The filtrate was extracted with two volumes of ethyl acetate and the organic layer

dried over anhydrous Na_2SO_4. The solvent was removed *in vacuo* at 30 C. Products were purified by column chromatography followed by recrystallization.

Whole Cell Studies

Determination of the rate of whole cell oxidation of various substrates was conducted polarographically with a Clark-type oxygen electrode at 30 C, using 0.05 M KH_2PO_4 buffer, pH 7.0, which was saturated with air prior to use. The reaction mixture, in a total volume of 1.4 ml contained buffer and an aliquot of a cell suspension. The reaction was initiated with the addition of 200 nmoles of the substrate in 10 µl of N,N-dimethylformamide. All rates were corrected for endogenous respiration of the cell suspension. Whole cells were unable to oxidize N,N-dimethylformamide nor did this solvent inhibit enzymatic activity.

Preparation of Cell Extracts

Frozen cells were thawed and suspended in 0.05 M KH_2PO_4 buffer, pH 7.2, containing 10% acetone (3 g of cells per 10 ml of buffer). The suspension was subjected to sonic oscillation at 40 kHz with a Bronwill Biosonik III ultrasonic disintegrator for 1.5 min (three 30-sec exposures). Cell debris was removed after centrifugation at 30000 x g for 1 hour at 5 C. The clear supernatant solution was used as a source of crude cell extract.

Enzyme Analyses

The activity of various enzymes was determined with cell-free extracts. In all cases, the amount of cell extract used in the analysis was a level found to give a linear rate of product formation with respect to protein concentration.

1,2-Dihydroxynaphthalene Oxygenase--The activity of the 1,2-dihydroxynaphthalene oxygenase was determined polarographically at 30 C using

0.05 M KH_2PO_4 buffer, pH 7.0. The reaction vessel, in a total volume of 1.4 ml contained buffer and a suitable amount of cell extract. Reactions were initiated with the addition of 100 nmoles of 1,2-dihydroxynaphthalene.

2,3-Dihydroxybiphenyl Oxygenase--Activity of the 2,3-dihydroxybiphenyl oxygenase was determined polarographically at 30 C using 0.05 M KH_2PO_4 buffer, pH 7.5. The reaction mixture, in a total volume of 1.4 ml contained buffer and a suitable amount of cell extract. Reactions were initiated with the addition of 100 nmoles of 2,3-dihydroxybiphenyl in 10µl of N,N-dimethylformamide. All rates were corrected for endogenous respiration. No auto-oxidation of 2,3-dihydroxybiphenyl was observed under the assay conditions.

Spectrophotometric assay conditions were established for the 2,3-dihydroxybiphenyl oxygenase. The product of the reaction has been identified as 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (5) and was found to exhibit an absorption maximum at 435 nm (ε = 15800; pH 7.5) (D.T. Gibson, unpublished results). The reaction mixture, in a total volume of 1.0 ml contained buffer and cell extract. Reactions were initiated with the addition of 100 nmoles of 2,3-dihydroxybiphenyl.

Catechol-2,3-dioxygenase--The activity of the catechol-2,3-dioxygenase (E.C. 1.13.1.2) was determined polarographically at 30 C using 0.05 M KH_2PO_4 buffer, pH 7.5. The reaction mixture, in a total volume of 1.4 ml contained buffer and a suitable amount of cell extract. Reactions were initiated with the addition of 1.0 µmole of catechol. All rates were corrected for endogenous respiration.

Catechol-2,3-dioxygenase activity was monitored spectrophotometrically by the procedure described by Nozaki et al. (22) by measuring the formation of α-hydroxymuconic semialdehyde at 375 nm. The reaction mixture, in a total

volume of 1.0 ml, contained buffer and cell extract. Reactions were initiated with the addition of 1.0 μmole of catechol.

Protein Determinations

Whole cell protein determinations were conducted after digestion of an aliquot of the cell suspension in 0.1 N NaOH at 100 C for 1 hour. Protein concentration was determined by the method of Lowry et al. (20) with crystalline bovine serum albumin as the standard.

Analytical Methods

Ultraviolet and visible spectra were determined on a Beckman model 25 recording spectrophotometer. Infrared spectra were recorded on a Perkin-Elmer model 137 spectrophotometer and were referenced to the absorptions of polystyrene. Crystalline samples were mulled in Nujol and placed between NaCl discs. Optical activity was determined with a Perkin-Elmer model 141 polarimeter. Low-resolution mass spectra were determined on a DuPont-Consolidated Electrodynamics Corp. model 21-491 spectrometer. High-resolution mass spectra were determined on a DuPont-Consolidated Electrodynamics Corp. model 21-1000C spectrometer and were referenced to assigned perfluoroalkane peak fragments. Proton magnetic resonance spectra were recorded on a Varian model HA-100 spectrometer. Absorptions were assigned δ values at the mid-point of half-height and are referenced to tetramethylsilane (Me_4Si). Melting points were obtained with the use of a Büchi melting point apparatus and are uncorrected.

Chemicals and Materials

Dibenzo-p-dioxin was synthesized according to the procedure described by Gilman and Dietrich (14). 1-Chloro- and 2-chlorodibenzo-p-dioxin were prepared by the method of Pohland and Yang (24). 2,3-Dichloro- and 2,7-dichlorodibenzo-p-dioxin were obtained from RFR Corp. 2,8-Dichloro- and 1,2,4-tri-

chlorodibenzo-p-dioxin were obtained from the Food and Drug Administration. 2-Hydroxydibenzo-p-dioxin was synthesized by a method analogous to that described by Hartough and Meisel (15) for the synthesis of 2-hydroxydibenzothiophene. Pyrocatechol was obtained from Eastman Kodak, Inc. 1,2-Dihydroxynaphthalene was synthesized by the method of Corner and Young (6). 2,3-Dihydroxybiphenyl was prepared as previously described (13). Oxidized nicotinamide adenine dinucleotide (NAD^+) was obtained from Sigma Chemical Co. Silica-Gel 60 was from Brinkman Instruments, Inc. Basic Alumina, Brockman Activity I was from Fisher Scientific Co. BioRad AG-1X8 was obtained from BioRad Laboratories. Diethylaminoethyl (DEAE)-cellulose was obtained from Whatman, Inc. Analytical t.l.c. was performed using Brinkman Chromatogram sheets, type Sil G/UV (Silica-Gel with fluorescent indicator). Preparative t.l.c. was conducted with glass plates (5.0 x 20.0 cm) precoated with Silica-Gel 60 (Brinkman Instruments, Inc.). Organic solvents were purified by vacuum distillation. All other materials were of the highest purity commercially available and were used without further purification.

SECTION 5

RESULTS AND DISCUSSIONS

Preliminary Studies

The initial approach was to attempt to isolate new microorganisms with the ability to degrade dibenzo-p-dioxin. The biodegradation of this compound would be studied in detail with respect to metabolites formed as well as the effect of the oxidation on the organism. However, numerous attempts to isolate microorganisms from freshwater and a wastewater treatment facility, capable of utilizing dibenzo-p-dioxin as a sole carbon source proved unsuccessful.

In a subsequent experiment, several different strains of bacteria, previously shown to be capable of oxidizing aromatic hydrocarbons, were screened for the ability to co-oxidize dibenzo-p-dioxin. Organisms were grown in 100 ml cultures of mineral salts broth with 0.2% succinate as the carbon source. Dibenzo-p-dioxin (0.05%) was added to the cultures as a solid. Growth of the experimental cultures was compared to the growth of control cultures grown on succinate (0.2%) in the absence of dibenzo-p-dioxin. The formation of products in the culture medium was analyzed qualitatively by thin-layer chromatography as described in Materials and Methods. Results are summarized in Table 1. Dibenzo-p-dioxin was unable to support the growth of any of the organisms when supplied as the sole carbon source. However, co-oxidation was observed under certain condtions. Among the strains tested, a Pseudomonas sp. and a Beijerinckia sp. were selected for further studies.

TABLE 1. SUMMARY OF THE PRELIMINARY SCREENING OF ORGANISMS FOR THE ABILITY TO CO-OXIDIZE DIBENZO-p-DIOXIN

Organism	Growth	Metabolite Formation	
		R_f[1]	Color Reaction[2]
Beijerinckia sp.	+		None detected
Beijerinckia sp. strain B8/36	++++	0.22 0.5	Brown Purple
Pseudomonas sp. N.C.I.B. 9816	++++		None detected
Pseudomonas sp. N.C.I.B. 9816 strain 11	++++	0.5 (trace)	Faint purple
P. putida NP	++++		None detected
P. putida NP strain 119	++++		None detected
P. putida TOL	++++		None detected
P. putida TOL strain 39/D	++++		None detected
Flavobacterium sp.	++++	0.5 (trace)	Faint purple
Mycobacterium sp.	+++		None detected
Acinetobacterium sp.	+++		None detected

[1]Chromatograms developed in $CHCl_3$:acetone (80/20)

[2]Color reaction with Gibbs reagent and subsequent exposure to ammonia vapors

Oxidation of Various Aromatic Hydrocarbons by *Pseudomonas* sp. N.C.I.B. 9816

Duplicate cultures (500 ml) of *Pseudomonas* sp. N.C.I.B. 9816 were grown to mid-log phase on the various carbon sources listed in Table 2. Oxygen consumption by washed, whole cells of *Pseudomonas* sp. N.C.I.B. 9816 was then examined in the presence of various aromatic substrates (Table 2). Cultures grown with succinate or on succinate with an inducing substrate exhibited little activity with the various substrates. Alternatively, cultures grown

TABLE 2. OXIDATION OF SUBSTRATES BY WASHED, WHOLE CELLS OF PSEUDOMONAS SP. N.C.I.B. 9816

Growth Substrate(s)	Oxygen uptake on various substrates (nmoles of O_2 consumed/$min^{-1}mg^{-1}$)				
	Naphthalene	Benzene	Anthracene	Dibenzo-p-dioxin	Catechol
0.2% Succinate	27.6	5.6	6.8	6.4	0.0
0.2% Succinate + 0.5% Salicylate	23.2	4.9	6.3	8.3	4.3
0.2% Succinate + 0.05% Naphthalene	19.2	5.8	5.2	8.9	4.8
0.2% Succinate + 0.05% Dibenzo-p-dioxin	9.1	0.0	0.0	0.0	0.0
0.1% Salicylate	294.0	21.1	36.8	21.1	215.4
0.1% Naphthalene	263.3	24.6	46.7	24.6	183.8

Enzymatic activity was monitored polarographically as described in Materials and Methods. Aromatic substrates were added in N,N-dimethylformamide (200 nmoles/10 µl).

with salicylate or naphthalene as the sole carbon source readily oxidized the various aromatic substrates. No attempt was made to isolate and identify the metabolites.

Oxidation of dibenzo-p-dioxin by Pseudomonas sp. N.C.I.B. 9816 strain 11

Preliminary studies of the oxidation of dibenzo-p-dioxin by Pseudomonas sp. N.C.I.B. 9816 strain 11 indicated that co-oxidation was negligible when the organism was grown with succinate (0.2%) as the carbon source. However, when Pseudomonas sp. N.C.I.B. 9816 strain 11 was grown in 500 ml cultures of mineral salts broth containing 0.1% salicylate and 0.05% dibenzo-p-dioxin, two neutral products (compounds A and B) were excreted into the culture medium (Fig. 1). Compound A (R_f 0.22) absorbed ultraviolet light and gave a brown color with Gibbs reagent. Compound B (R_f 0.5) also absorbed ultraviolet light and gave a purple color with Gibbs reagent. The formation of compound A in the culture medium was monitored spectrophotometrically (Figure 2). Maximum accumulation of compound A was observed after 36 hours.

It is of interest that Pseudomonas sp. N.C.I.B. 9816 strain 11 failed to co-oxidize dibenzo-p-dioxin when grown on succinate but readily oxidized the compound when grown on salicylate. This observation is consistent with the report by Shamsuzzaman and Barnsley (28) suggesting that in this organism, salicylate is the coordinate inducer of a block of enzymes catalyzing reactions in the conversion of naphthalene to salicylate. The results also indicate that dibenzo-p-dioxin fails to induce the enzymes responsible for the oxidation of the aromatic nucleus.

Isolation of Compounds A and B

Pseudomonas sp. N.C.I.B. 9816 strain 11 was grown in 10 liters of mineral salts broth containing 0.1% salicylate and 0.05% dibenzo-p-dioxin. After 36

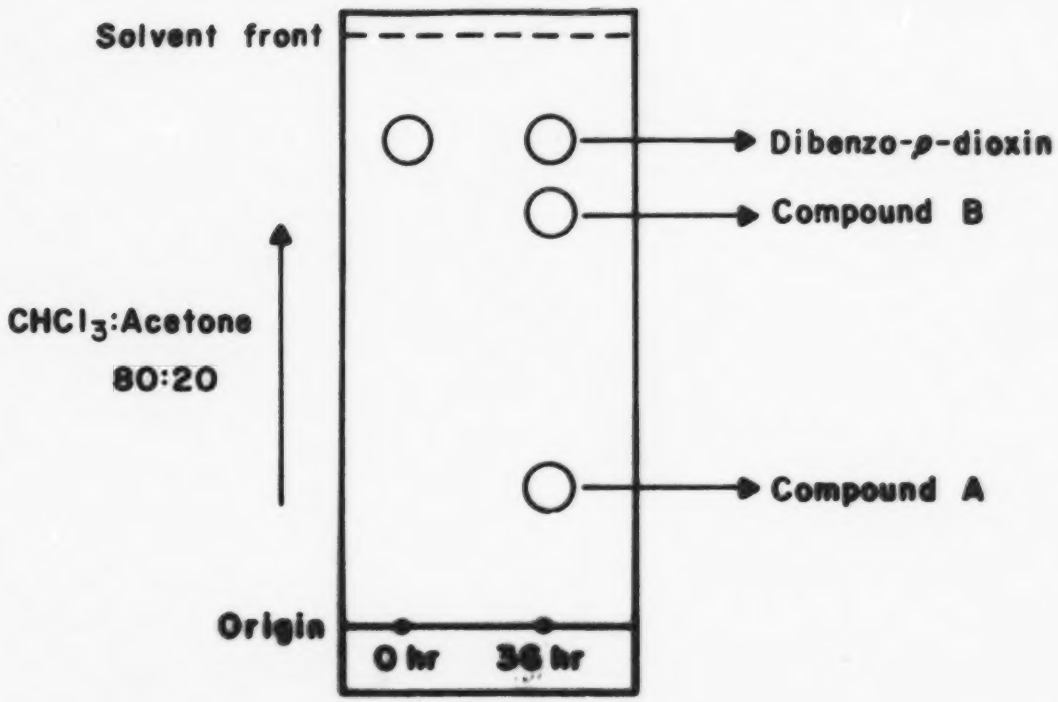


Figure 1. Thin-layer chromatogram of the products formed from the oxidation of dibenzo-p-dioxin by Pseudomonas sp. N.C.I.B. 9816 strain 11. Experimental details are discussed in the text.

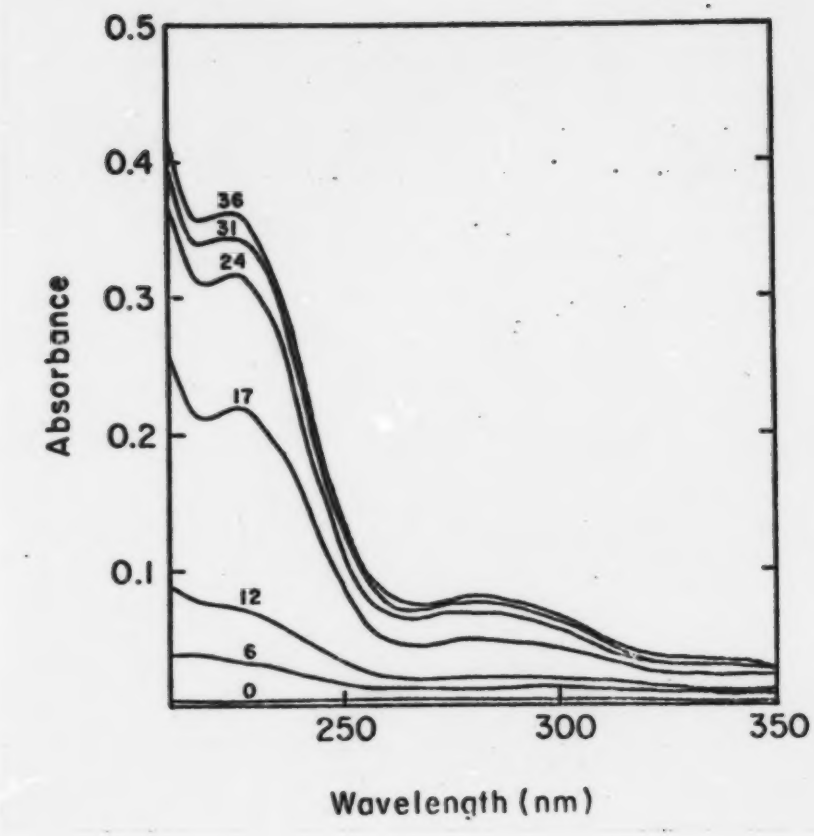


Figure 2. Formation of compound A by Pseudomonas sp. N.C.I.B. 9816 strain 11 (legend next page).

hours, the culture was filtered to remove unused dibenzo-p-dioxin and then extracted with an equal volume of ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4 and the solvent removed in vacuo at 35 C to yield 367 mg of a solid residue.

The residue was dissolved in a small volume of chloroform and applied to the top of a silica-gel column (1.9 x 22.0 cm; 25% H_2O w/v). The column was eluted with 700 mls of chloroform and then 700 mls of chloroform/methanol (98:2). The presence of compounds A and B in the column fractions (15 mls) was detected by thin-layer chromatography. Fractions 9-15 were found to contain compound B. Compound A was eluted in fractions 63-75. Fractions con-

Figure 2 Legend.

A 500 ml culture was grown as described in the text. At the times indicated (hours) the accumulation of compound A was measured quantitatively as described in Materials and Methods.

taining each metabolite were pooled and the solvent removed in vacuo at 30 C. Compound A (242 mg) and compound B (276 mg) were further purified by two recrystallizations from hexane/acetone.

Identification of Compound B

The white crystals obtained after recrystallization had the following physical properties: melting point, 144-145 C; mass spectrum, calculated for $^{12}C_{12}{}^{1}H_{8}{}^{16}O_{3}$, 200.0473, found mass, 200.0468. The infrared spectrum (Nujol) exhibited maxima at 2.82, 2.92, 6.15, 6.32, 7.53, 10.83, 12.5, and 13.4 μm (Figure 3). The ultraviolet spectrum (MeOH) showed maxima at 221 nm (ε = 32000) and 292 nm (ε = 4300) (Figure 4). The proton magnetic resonance spectrum of compound B in deuterated acetone is shown in Figure 5. An analysis of the spectrum is given in Table 3.

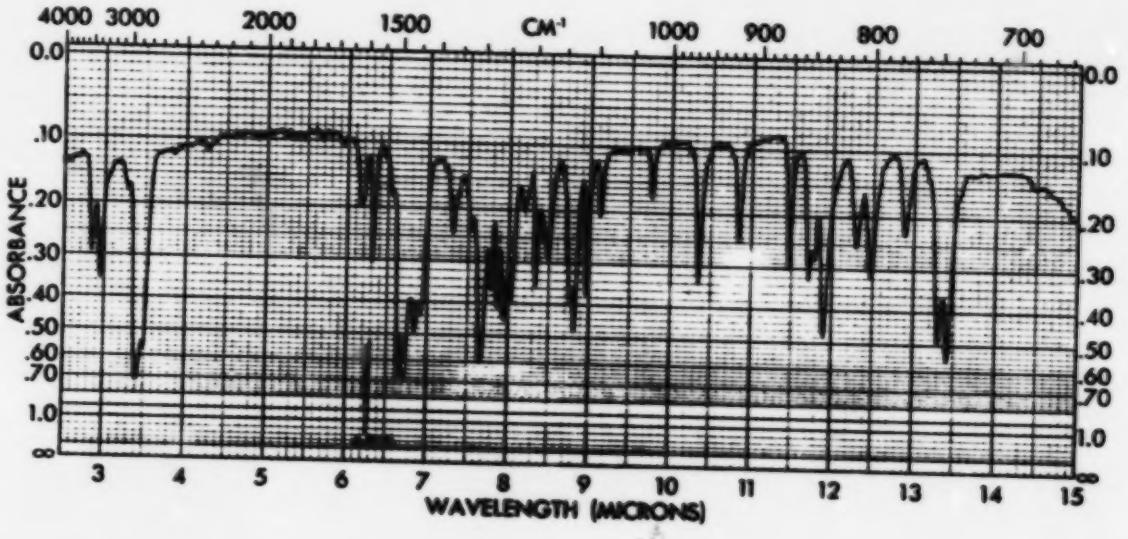


Figure 3. Infrared spectrum of compound B (Nujol)

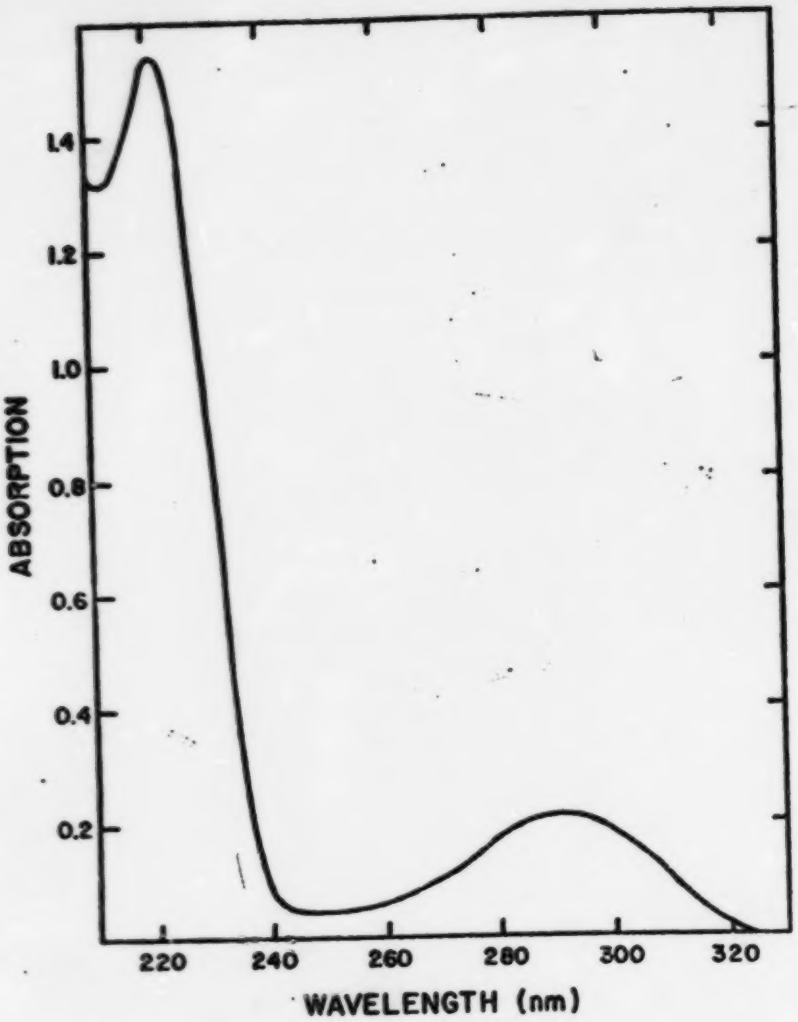


Figure 4. Ultraviolet spectrum of compound B (MeOH)

The results of the physical data suggest that compound B is 2-hydroxydibenzo-p-dioxin. Verification of this structure was obtained with the chemical synthesis of 2-hydroxydibenzo-p-dioxin from 2-bromodibenzo-p-dioxin via an alkali fusion reaction. Compound B exhibited infrared, ultraviolet and mass spectra identical to those of the synthetic compound. No depression of melting point was observed when compound B was mixed with the authentic sample of 2-hydroxydibenzo-p-dioxin.

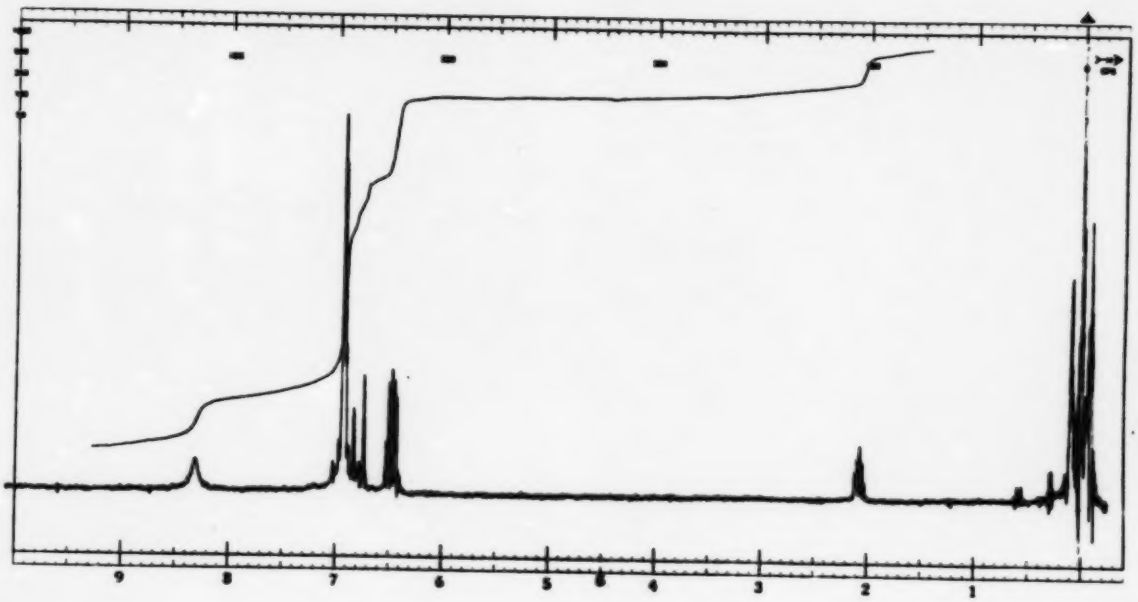
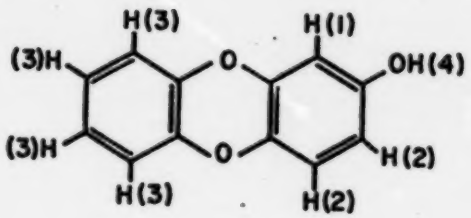


Figure 5. Proton magnetic resonance spectrum of compound B (Acetone-d_6)

TABLE 3. ANALYSIS OF THE PROTON MAGNETIC RESONANCE SPECTRUM OF COMPOUND B



Proton	Chemical Shift (δ)	Description
1	6.77	1H [m, aromatic]
2	6.46	2H [m, aromatic]
3	6.82-7.02	4H [m, aromatic]
4	8.20-8.44	1H [b, hydroxyl]

Identification of Compound A

The white crystals obtained after recrystallization had the following physical properties: melting point, 139-140 C; $[\alpha]_D^{25}$ + 110° (c = 0.405, MeOH); mass spectrum, calculated for $^{12}C_{12}{}^{1}H_{10}{}^{16}O_4$, 218.0579, found mass, 218.0587. The infrared spectrum (Nujol) showed peaks at 3.05, 5.92, 6.3, 6.9, 7.95, 10.82, 12.0, 12.4, and 14.18 μm (Figure 6). The ultraviolet spectrum in methanol (Figure 7) exhibited maxima at 227 nm (ε = 12600) and 298 nm (ε = 2800). The proton magnetic resonance spectrum of compound A in deuterated pyridine is shown in Figure 8. An analysis of the spectrum is given in Table 4.

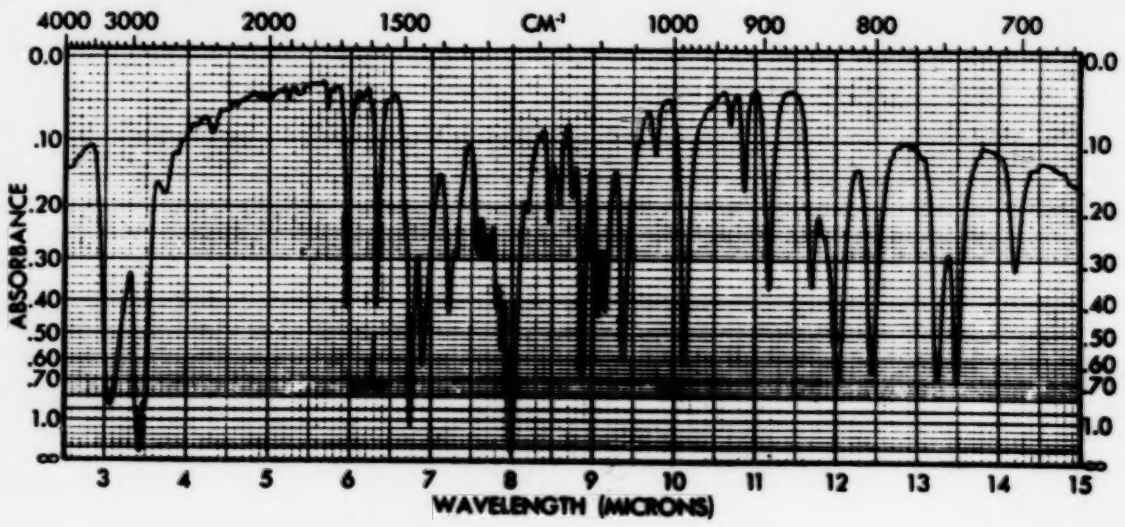


Figure 6. Infrared spectrum of compound A (Nujol)

The physical data suggest that compound A is 1,2-dihydroxy-1,2-dihydro-dibenzo-p-dioxin. Additional support for the assigned structure was provided by the acid-catalyzed dehydration of compound A. Compound A (53 mg) was dissolved in 20 ml of ethyl ether. Concentrated HCl (5.0 μl) was added to the solution. After 1 hour of incubation at 27 C the solvent was removed,

leaving 46 mg of a solid residue. The physical properties of the resulting compound were found to be identical with compound B which was previously identified as 2-hydroxydibenzo-p-dioxin.

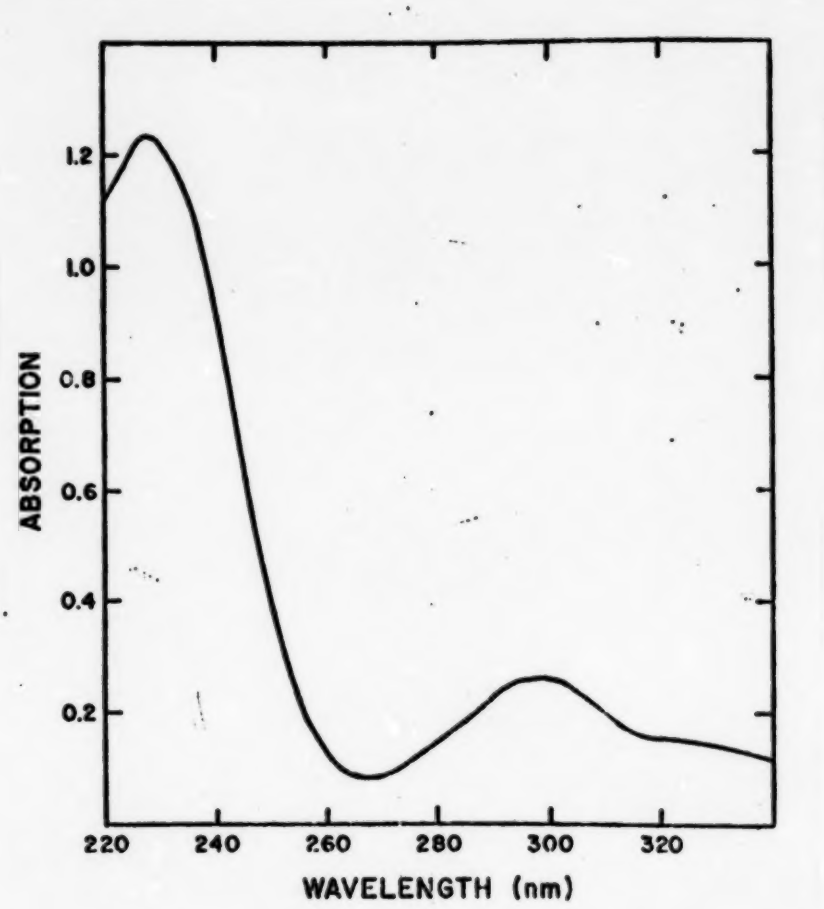


Figure 7. Ultraviolet spectrum of compound A (MeOH)

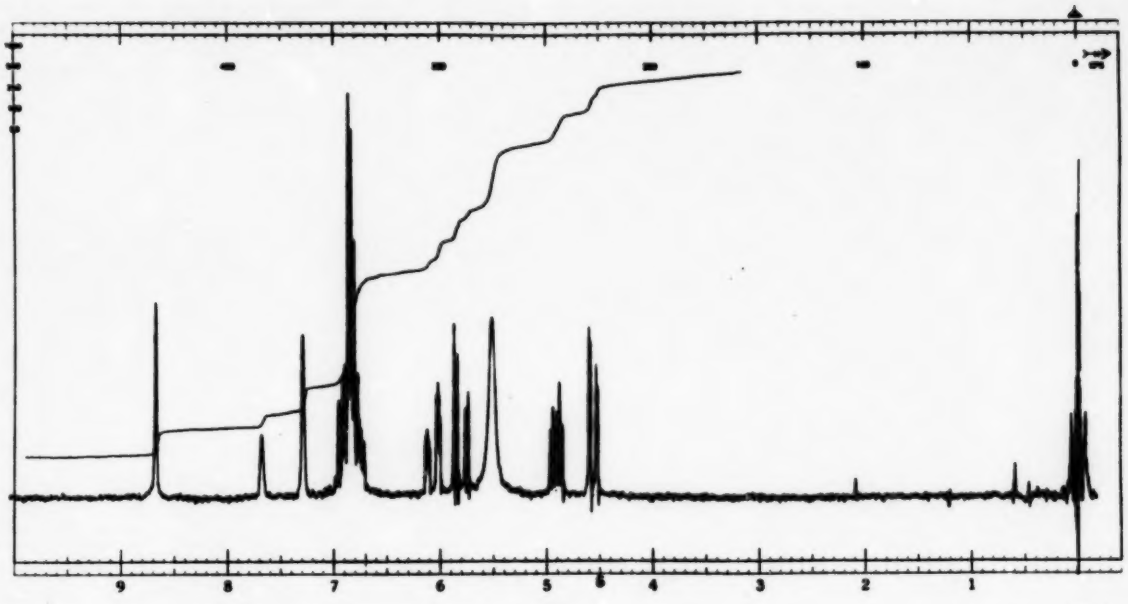
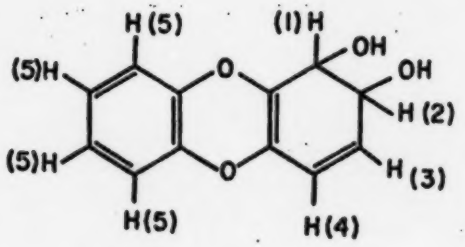


Figure 8. Proton magnetic resonance spectrum of compound A (Pyridine-d_5). The hydroxyl protons were exchanged with D_2O before recording the spectrum as they interferred with the signal given by H(5).

TABLE 4. ANALYSIS OF THE PROTON MAGNETIC RESONANCE SPECTRUM OF COMPOUND A



Proton	Chemical Shift (δ)	Description
1	4.55	1H [d, hydroxymethine, $J_{1,2}$ = 6.5 Hz]
2	4.92	1H [m, hydroxymethine, $J_{2,1}$ = 6.5 Hz]
3	5.80	1H [dd, olefinic, $J_{3,4}$ = 10.0 Hz $J_{3,2}$ = 2.0 Hz]
4	6.06	1H [m, olefinic, $J_{4,3}$ = 10.0 Hz]
5	6.70-7.00	4H [m, aromatic]

Although the results obtained establish the structure of compound A as 1,2-dihydroxy-1,2-dihydrodibenzo-p-dioxin, they do not distinguish between the possible cis and trans stereoisomers. Evidence for a cis configuration was obtained by the synthesis of an isopropylidine derivative (3). Compound A (290 mg) was suspended in 20 ml of 2,2-dimethoxypropane. The suspension was chilled in an ice bath prior to the addition of 1.0 mg of p-toluenesulfonic acid. The reaction was monitored by thin-layer chromatography. After 4 hours the presence of compound A in the reaction mixture could no longer be detected, however, a nonpolar compound (compound C; R_f 0.64) was observed that absorbed ultraviolet light and gave a yellow-brown color with Gibbs reagent. At this point, triethylamine (0.2 ml) was added to neutralize the acid and the solvent was removed in vacuo at 35 C. The oily residue (335 mg) was dissolved in a small volume of hexane and applied to the top of a column of basic alumina (1.0 x 16.0 cm). Elution with benzene/hexane (1:1) yielded 90 mg of a white solid. Compound C had the following physical properties: melting point, 97-99 C; $[\alpha]_D^{25}$ + 232° (c = 0.41 in methanol); mass spectrum, calculated for $^{12}C_{15}{}^{1}H_{14}{}^{16}O_4$, 258.0892, found mass 258.0886. The infrared spectrum (Nujol) showed maxima at 5.85, 6.27, 6.93, 7.93, 7.95, 8.67, 8.9, 10.8, 12.7, and 13.4 μm (Figure 9). The ultraviolet spectrum in methanol (Figure 10) exhibited absorption maxima at 228 nm (ε = 13900) and 297 nm (ε = 2300). The proton magnetic resonance spectrum is shown in Figure 11. An analysis of the proton magnetic resonance spectrum of compound C is given in Table 5. These observations establish the structure of compound C as 1,3-dioxolo-2,2-dimethyl-1,2-dihydrodibenzo-p-dioxin.

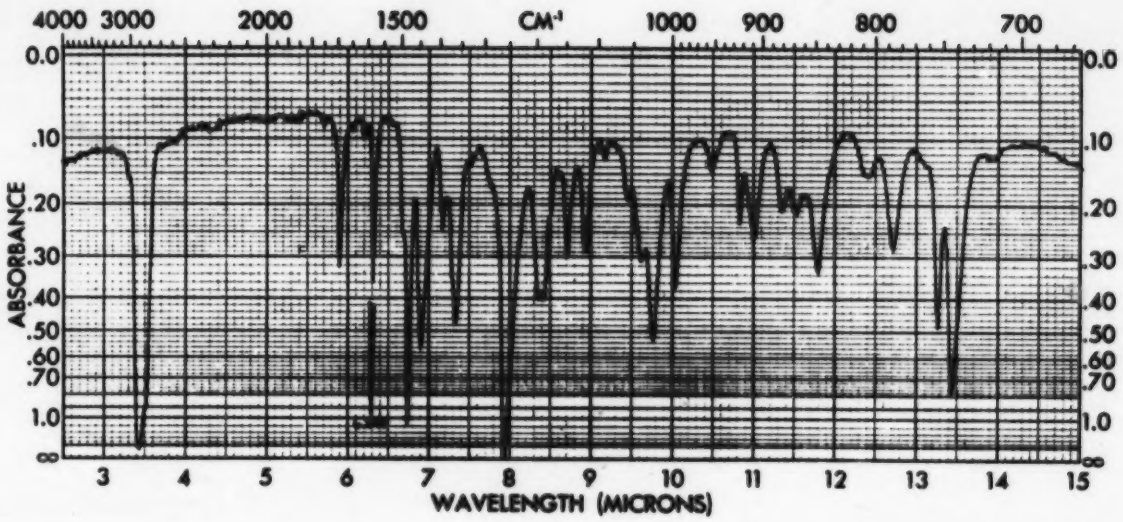


Figure 9. Infrared spectrum of compound C (Nujol)

The present study has demonstrated that cis-1,2-dihydroxy-1,2-dihydrodibenzo-p-dioxin is the first detectable metabolite in the oxidation of dibenzo-p-dioxin by a Pseudomonas sp. It should be noted that the cis-stereochemistry of the hydroxyl groups was inferred by the formation of an isopropylidene derivative with 2,2-dimethoxypropane (3) and also from the fact that cis-dihydrodiols have been implicated as intermediates in the bacterial metabolism of a variety of aromatic hydrocarbons (11). The cis-dibenzo-p-dioxin dihydrodiol can subsequently undergo acid-catalyzed dehydration to yield 2-hydroxydibenzo-p-dioxin.

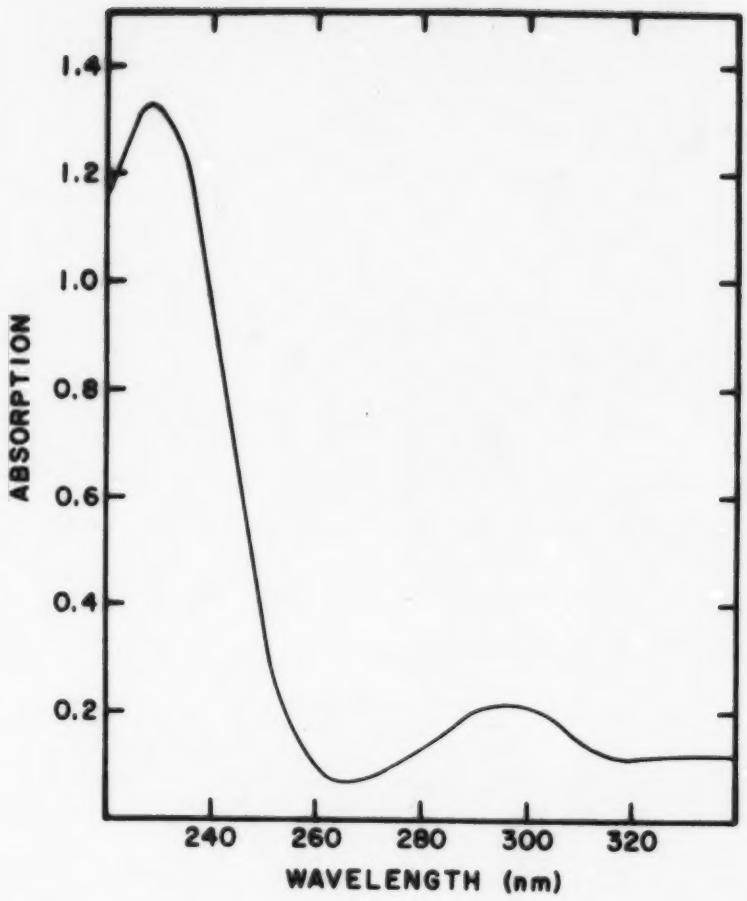


Figure 10. Ultraviolet spectrum of compound C (MeOH)

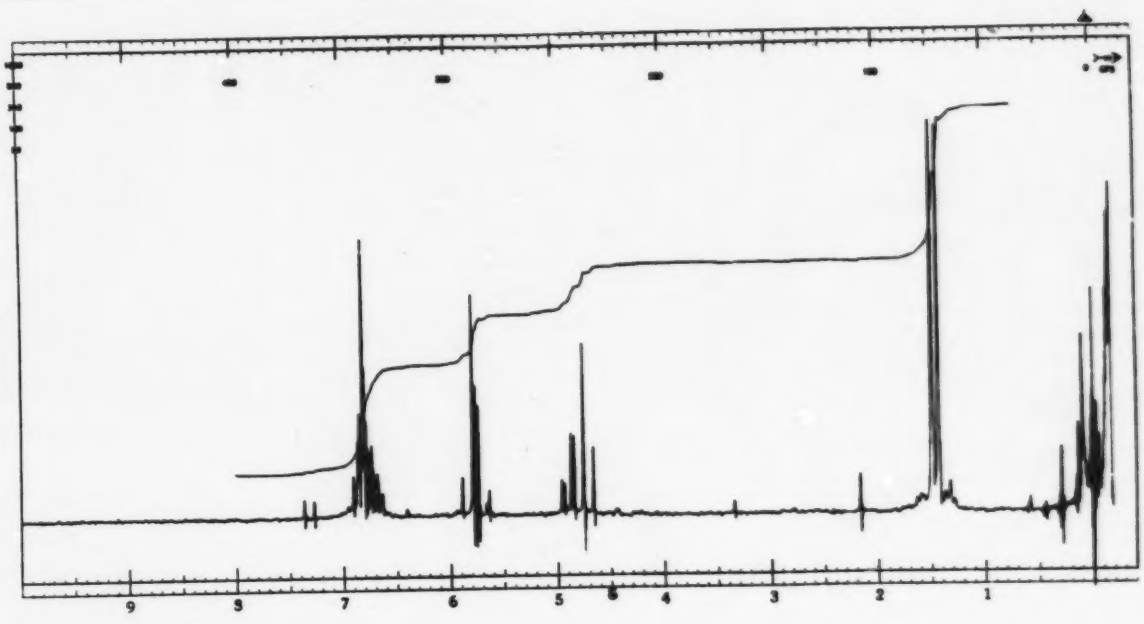
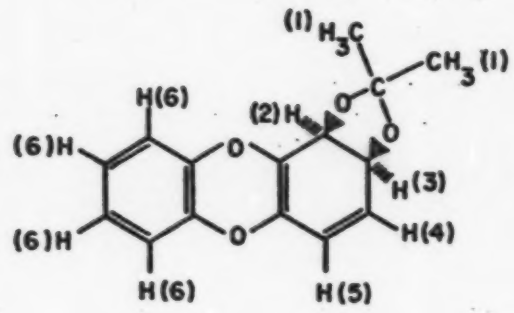


Figure 11. Proton magnetic resonance spectrum of compound C ($CDCl_3$)

TABLE 5. ANALYSIS OF THE PROTON MAGNETIC RESONANCE SPECTRUM OF COMPOUND C



Proton	Chemical Shift (δ)	Description
1	1.44	3H [s, methyl]
1	1.49	3H [s, methyl]
2	4.71	1H [d, alkoxymethine, $J_{2,3}$ = 8.0 Hz]
3	4.90	1H [dd, alkoxymethine, $J_{3,2}$ = 8.0 Hz, $J_{3,4}$ = 3.0 Hz]
4	5.68	1H [dd, olefinic, $J_{4,5}$ = 10.0 Hz, $J_{4,3}$ = 3.0 Hz]
5	5.83	1H [d, olefinic, $J_{5,4}$ = 10.0 Hz]
6	6.62-6.90	4H [m, aromatic]

Metabolism of 1,2-dihydroxy-1,2-dihydrodibenzo-p-dioxin by cell extracts

When cell extracts prepared from the parent organism were incubated under anaerobic conditions with cis-1,2-dihydroxy-1,2-dihydrodibenzo-p-dioxin and NAD^+, a product of the reaction (compound D) was detected by thin-layer chromatography (R_f 0.34) that absorbed ultraviolet light and gave an intense brown color with Gibbs reagent. In a large-scale reaction, crude cell extract (80 mg of protein) was mixed with cis-1,2-dihydroxy-1,2-dihydrodibenzo-p-dioxin (0.7 mmoles) and NAD^+ (1.0 mmoles) in 40 ml of 0.05 M KH_2PO_4 buffer, pH 7.2, and incubated under anaerobic conditions in a sealed serum bottle. After 3 hours, the reaction mixture was acidified with 0.5 ml of 5 N HCl and extracted with three volumes of ethyl acetate. The organic extracts were pooled, dried over anhydrous Na_2SO_4, and the solvent removed in vacuo at 35 C to yield 185 mg of a solid residue. The residue was suspended in a small volume of chloroform and loaded onto the top of a silica-gel column (1.0 x 20.0 cm; 25% H_2O). The column was eluted with 500 mls of chloroform and 10 ml fractions were collected. The presence of compound D in fractions 16-28 was detected by thin-layer chromatography. These fractions were pooled and the solvent removed to yield 90 mg of compound D which was further purified by two recrystallizations from hexane/acetone.

Identification of Compound D

The white crystals obtained after recrystallization had the following physical properties: melting point, 172-173 C; mass spectrum, calculated for $^{12}C_{12}{}^{1}H_{8}{}^{16}O_4$, 216.0422, found mass, 216.0422. The infrared spectrum (Nujol) showed maxima at 2.85, 3.06, 6.15, 6.3, 7.73, 10.8, 12.72, and 13.4 μm (Figure 12). The ultraviolet spectrum in methanol (Figure 13) exhibited maxima at 229 nm (ε = 30800) and 289 nm (ε = 3300). The proton magnetic resonance

spectrum of compound D in deuterated acetone is shown in Figure 14. An analysis of the spectrum is given in Table 6. Due to a rapid exchange rate the hydroxyl protons were not observed at ambient temperature, however they were observed at 8.65 δ when the spectrum was recorded at -15 C.

The results of the physical data suggest that compound D is 1,2-dihydroxydibenzo-p-dioxin. This observation is consistent with the fact that several cis-dihydrodiols have been shown to be oxidized to catechols by pyridine nucleotide-dependent dehydrogenases (1,23,25,26).

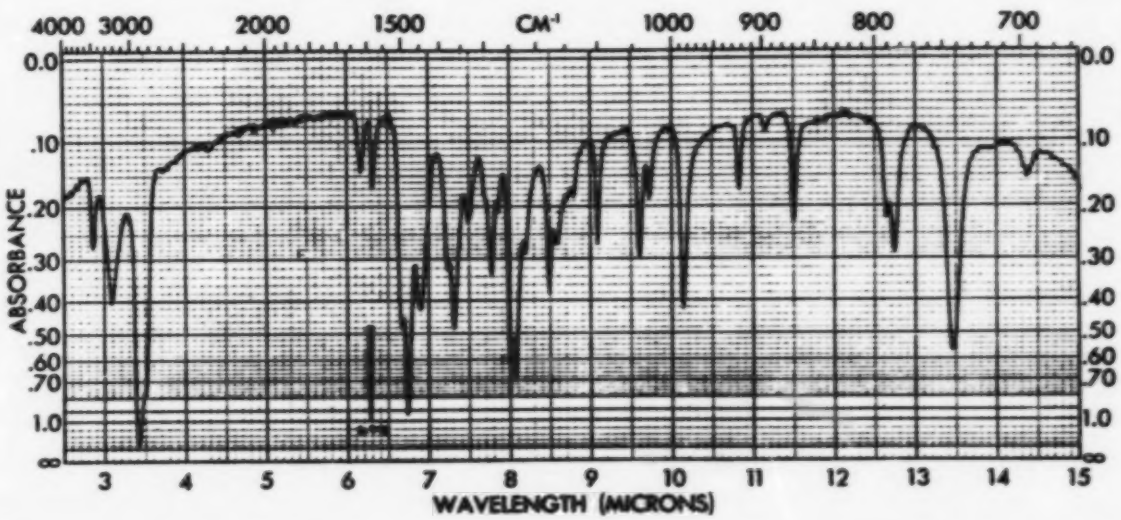


Figure 12. Infrared spectrum of compound D (Nujol)

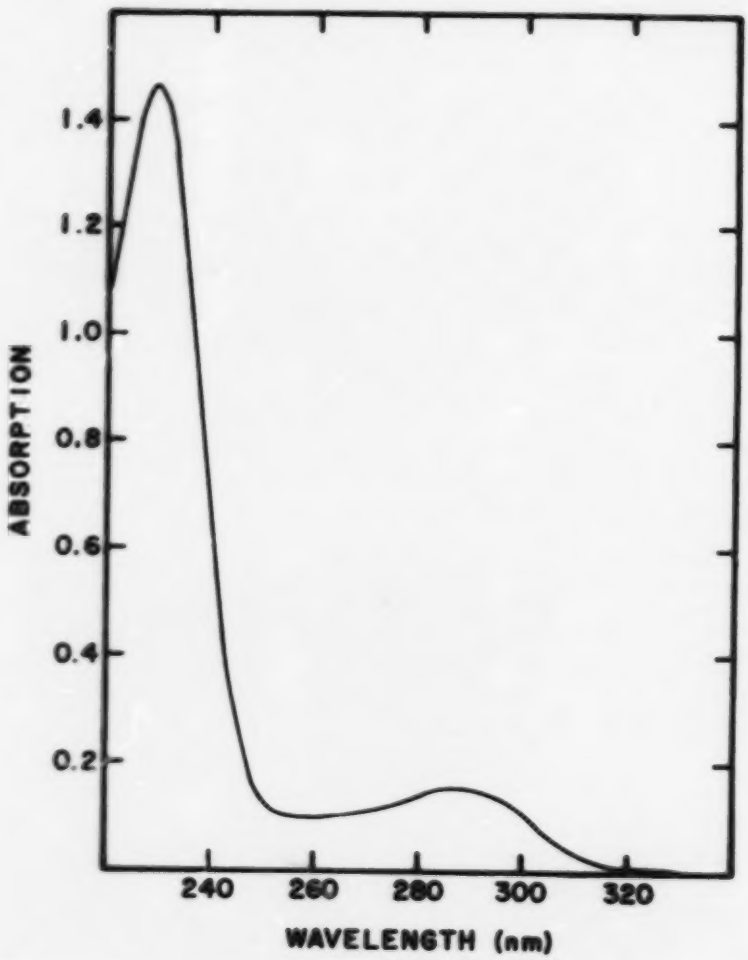


Figure 13. Ultraviolet spectrum of compound D (MeOH)

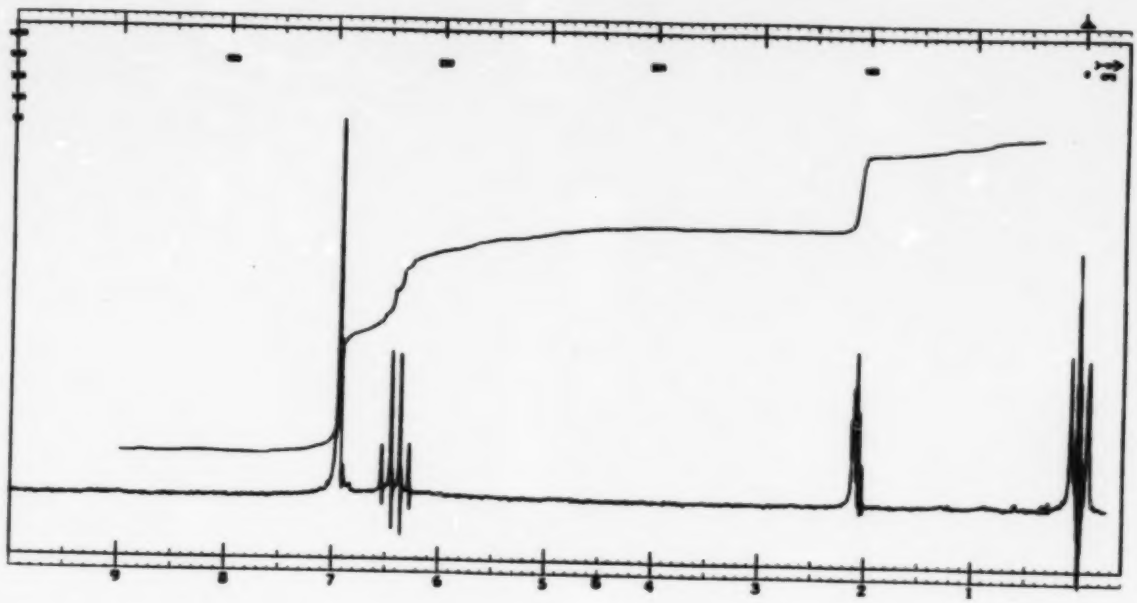
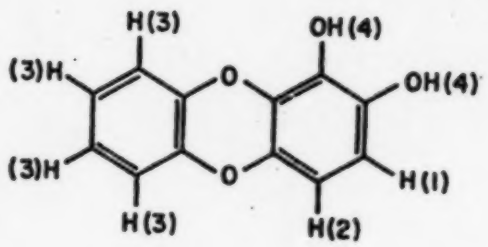


Figure 14. Proton magnetic resonance spectrum of compound D (Acetone-d_6)

Oxidation of 1,2-dihydroxydibenzo-p-dioxin by cell extracts

Polarographic experiments were performed to test the ability of 1,2-dihydroxy-dibenzo-p-dioxin to serve as a substrate for the 1,2-dihydroxynaphthalene oxygenase in extracts of the parent strain of Pseudomonas sp. N.C.I.B. 9816 (Figure 15). An initial investigation revealed that cell extracts were extremely active on the physiological substrate 1,2-dihydroxynaphthalene (4724 nmoles of O_2 consumed/min per mg of protein), however only slight activity was observed with 1,2-dihydroxydibenzo-p-dioxin (54 nmoles of O_2 consumed/min per mg of protein). It was also noted that incubation of cell extracts (0.15 mg of protein) with as low as 10 nmoles of 1,2-dihydroxydibenzo-p-dioxin led to the complete inhibition or inactivation of the 1,2-dihydroxynaphthalene oxygenase, as additions of 1.0 µmole of 1,2-dihydroxynaphthalene did not result in further enzymatic activity.

TABLE 6. ANALYSIS OF THE PROTON MAGNETIC RESONANCE SPECTRUM OF COMPOUND D



Proton	Chemical Shift (δ)	Description
1	6.45	1H [d, aromatic $J_{1,2}$ = 8.0 Hz]
2	6.52	1H [d, aromatic $J_{2,1}$ = 8.0 Hz]
3	6.88-7.08	4H [m, aromatic]
4	8.25-8.96	2H [b, hydroxyl]

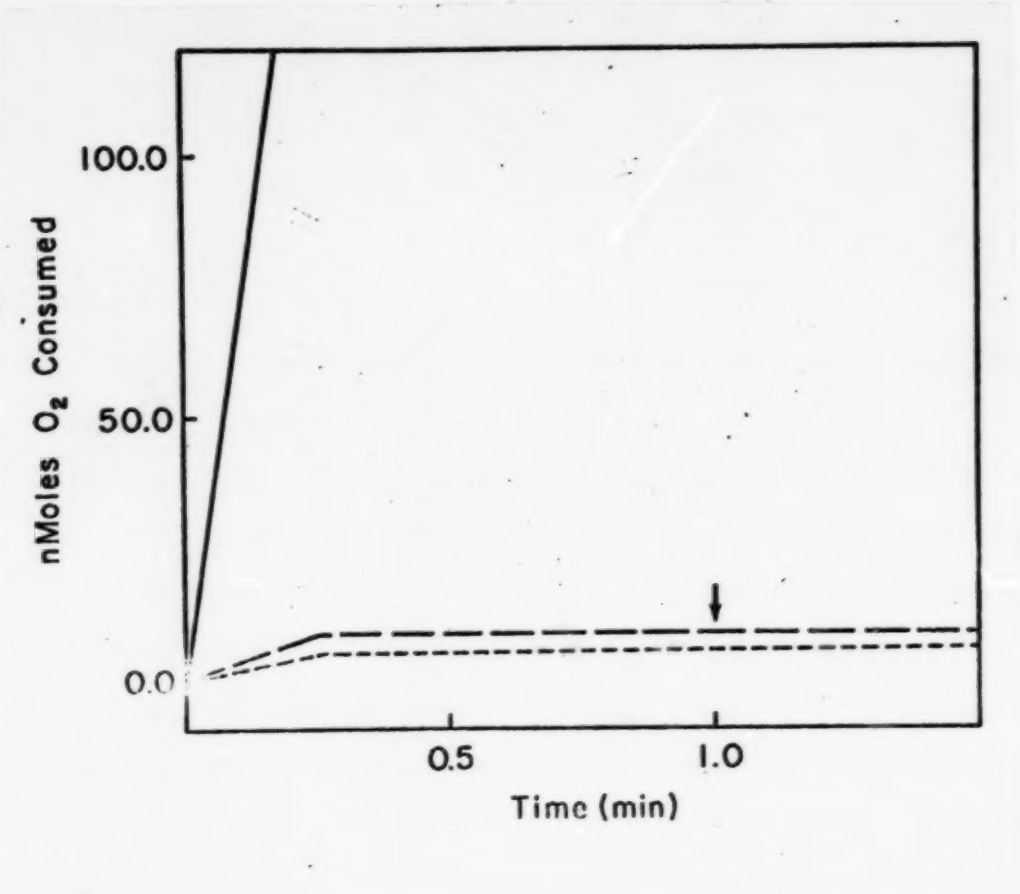


Figure 15. Polarographic assay of 1,2-dihydroxynaphthalene oxygenase in cell-free extracts of Pseudomonas sp. N.C.I.B. 9816.

Oxidation of dibenzo-p-dioxin and chlorinated dibenzo-p-dioxins by Beijerinckia sp.

Duplicate cultures (500 ml) of the parent strain of Beijerinckia sp. were grown to mid-log phase in mineral salts broth on succinate with biphenyl as an inducing substrate. In addition, an anion-exchange resin (AG 1-X8; 5.0 g/l) was included to remove acid products (19). The ability of whole cells of Beijerinckia sp. to oxidize dibenzo-p-dioxin and several chlorinated dibenzo-p-dioxins was investigated. The rate of oxidation of the dibenzo-p-dioxins was compared to the ability of the organism to oxidize biphenyl. Results of

Figure 15 Legend.

Polarographic assay of 1,2-dihydroxynaphthalene oxygenase in cell-free extracts of Pseudomonas sp. N.C.I.B. 9816.

Activity of the 1,2-dihydroxynaphthalene oxygenase was monitored polarographically as described in Materials and Methods. A protein concentration of 0.15 mg was used in the analysis. The reaction was initiated with the addition of; 100 nmoles of 1,2-dihydroxynaphthalene in 10 µl of anhydrous tetrahydrofuran (————), 10 nmoles of 1,2-dihydroxydibenzo-p-dioxin in 10 µl of anhydrous tetrahydrofuran (---------), and 100 nmoles of 1,2-dihydroxydibenzo-p-dioxin in 10 µl of anhydrous tetrahydrofuran (— — —). At the point indicated by the arrow 1.0 µmole of 1,2-dihydroxynaphthalene (in 10 µl of anhydrous tetrahydrofuran) was added. All rates were corrected for endogenous respiration and for the nonenzymatic oxidation of 1,2-dihydroxynaphthalene. No auto-oxidation of 1,2-dihydroxydibenzo-p-dioxin was observed under the assay conditions described.

the investigation are summarized in Table 7. Dibenzo-p-dioxin was oxidized at a lower rate than the monochlorinated dibenzo-p-dioxins. The fact that monochlorinated dibenzo-p-dioxins were oxidized faster than the parent compound may reflect substrate or product inhibition of the enzymes systems involved. Further studies are needed to clarify this point. The rate of oxidation of the chlorinated dibenzo-p-dioxins decreased with an increasing degree of chlorine substitution.

TABLE 7. OXIDATION OF SUBSTRATES BY WASHED, WHOLE CELLS OF BEIJERINCKIA SP.

Substrate[1]	Specific activity[2]	Relative rate (%)
Biphenyl	72.3	100.0
Dibenzo-p-dioxin	29.8	41.2
1-Chlorodibenzo-p-dioxin	52.5	72.6
2-Chlorodibenzo-p-dioxin	52.6	72.7
2,3-Dichlorodibenzo-p-dioxin	11.2	15.5
2,7-Dichlorodibenzo-p-dioxin	8.5	11.8
2,8-Dichlorodibenzo-p-dioxin	8.5	11.8
1,2,4-Trichlorodibenzo-p-dioxin	6.5	8.9

[1]All substrates added in N,N-dimethylformamide (200 nmoles/10.0 µl)

[2]Oxygen uptake at 30 C (nmoles of O_2 consumed/min per mg of protein)

Oxidation of dibenzo-p-dioxin (I) by Beijerinckia B8/36

When Beijerinckia B8/36 was grown in mineral salts medium containing 0.2% succinate and 0.05% dibenzo-p-dioxin (I) a neutral product (compound IA) was

detected in the culture medium. Compound IA (R_f 0.22) absorbed ultraviolet light and gave a brown color with Gibbs reagent. Growth of the culture and accumulation of compound IA with time are shown in Figure 16. After 24 hours, duplicate cultures were extracted to leave 5.8 mg of a solid residue. Purification of the residue led to the isolation of 3.0 mg of a crystalline product that melted at 135-136 C (compound IA). The metabolite had mass, infrared and ultraviolet spectra identical to those of 1,2-dihydroxy-1,2-dihydrodibenzo-p-dioxin. This product was previously obtained from the co-oxidation of dibenzo-p-dioxin by Pseudomonas sp. N.C.I.B. 9816 strain 11. Further support for this structure was provided by the conversion of compound IA to 2-hydroxydibenzo-p-dioxin. Compound IA (2.0 mg) was suspended in 3.0 ml of methanol and the reaction was initiated with the addition of 10.0 µl of 5.0 N H_2SO_4. After 30 min incubation at 27 C the solvent was removed, leaving 2.0 mg of a solid residue. Thin-layer chromatography of the residue revealed that compound IA was converted to a phenolic product (compound IB; R_f 0.5) that gave a purple color with Gibbs reagent. The latter compound was also detected in the co-oxidation cultures after 20 h. Compound IB was identified as 2-hydroxydibenzo-p-dioxin, having a melting point, mass, infrared and ultraviolet spectra identical to those of the known standard. Preliminary evidence for a cis-configuration of compound IA (1.0 mg) was suspended in 3.0 ml of 2,2-dimethoxypropane and the reaction was initiated with the addition of 0.1 mg of p-toluenesulfonic acid. Analysis of the reaction mixture by thin-layer chromatography revealed the formation of a nonpolar product (compound IC; R_f 0.64) that absorbed ultraviolet light and gave a yellow-brown color with Gibbs reagent. These results indicate that cis-1,2-dihydroxy-1,2-dihydrodibenzo-p-dioxin is the first detectable metabolite of the oxidation of dibenzo-p-dioxin by

Beijerinckia sp. This compound can subsequently undergo a non-enzymatic dehydration to yield 2-hydroxydibenzo-p-dioxin.

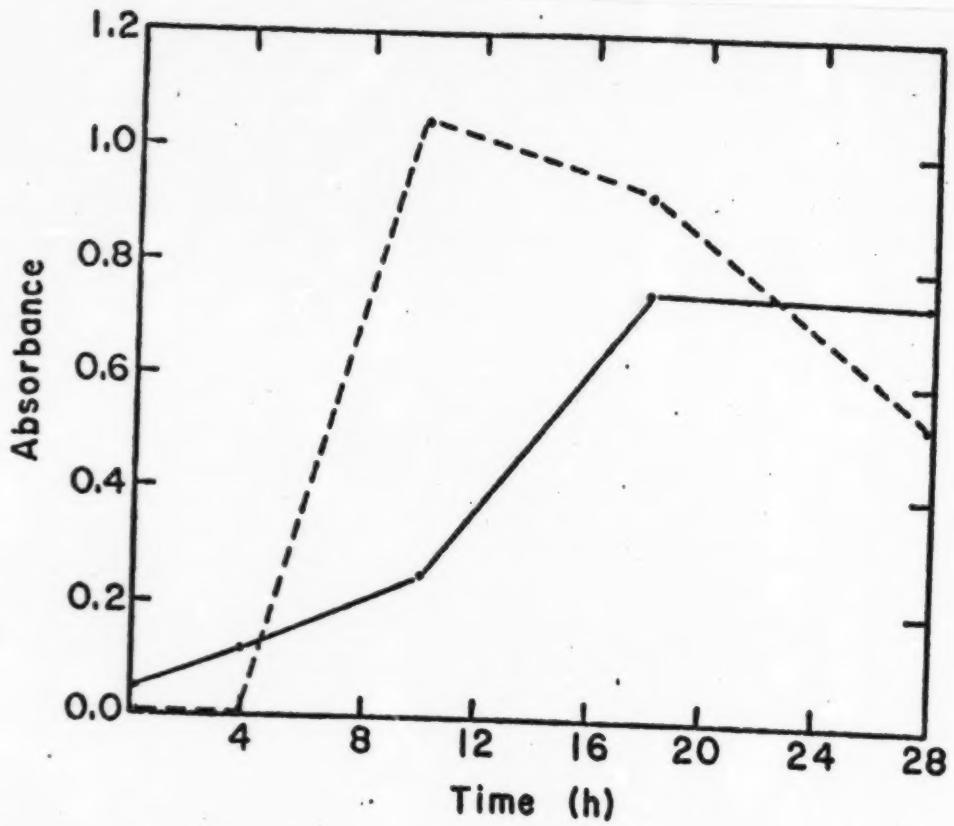


Figure 16. Growth and accumulation of compound IA by cultures of Beijerinckia B8/36. (legend next page).

Oxidation of chlorinated dibenzo-p-dioxins by Beijerinckia B8/36

Beijerinckia B8/36 was grown in duplicate cultures as above with the exception that dibenzo-p-dioxin was replaced by 1-chloro- (II), 2-chloro- (III),

Figure 16 Legend.

A 500 ml culture was grown as described in the text. Growth of the culture was monitored turbidometrically at 600 nm (————). Accumulation of compound IA was measured quantitatively as described in Materials and Methods. Analysis of the ultraviolet spectrum of compound IA indicated an absorption maximum at 227 nm. Accumulation of compound IA with time is expressed as A_{227} per ml of culture (----------).

2,3-dichloro- (IV), and 2,7-dichloro- (V) dibenzo-p-dioxins. The accumulation of neutral products (compounds IIA and IIIA) was detected in cultures containing monochlorinated dibenzo-p-dioxins (Figure 17). No products were detected in cultures grown in the presence of two dichlorinated dibenzo-p-dioxins.

Cultures containing monochlorinated dibenzo-p-dioxins were extracted with ethyl acetate. Subsequent purification led to the isolation of 2.5 mg and 1.5 mg of compounds IIA and IIIA, respectively. The properties of the crystalline products are summarized in Table 8. The results indicate that the monochlorinated dibenzo-p-dioxins were oxidized to dihydrodiols. These results also indicate that dibenzo-p-dioxin and monochlorinated dibenzo-p-dioxins induce the synthesis of enzymes responsible for oxidation of the aromatic nucleus in the Beijerinckia sp. Furthermore, it appears that the dichlorinated dibenzo-p-dioxins fail to function as inducing substrates. Thus, the degree of chlorine substitution on the aromatic ring may have an effect on the ability of a compound to induce the synthesis of degradative enzymes in this organism. In contrast, previous studies have demonstrated that dibenzo-p-dioxin failed to induce the synthesis of enzymes necessary for aromatic hydrocarbon degradation in Pseudomonas sp. N.C.I.B. 9816.

Further support for the formation of dihydrodiols was indicated by the acid-catalyzed dehydration of compounds IIA and IIIA to phenols (Figure 18). Compound IIA was converted to a product (compound IIB; R_f 0.5) that absorbed ultraviolet light and gave a purple color with Gibbs reagent. Compound IIIA was also converted to a phenolic product (compound IIIB; R_f 0.51) that absorbed ultraviolet light and gave a dark blue color with Gibbs reagent. Additional products were not detected in the reactions mixtures. It is of interest that the acid-catalyzed dehydration of the dibenzo-p-dioxin and the

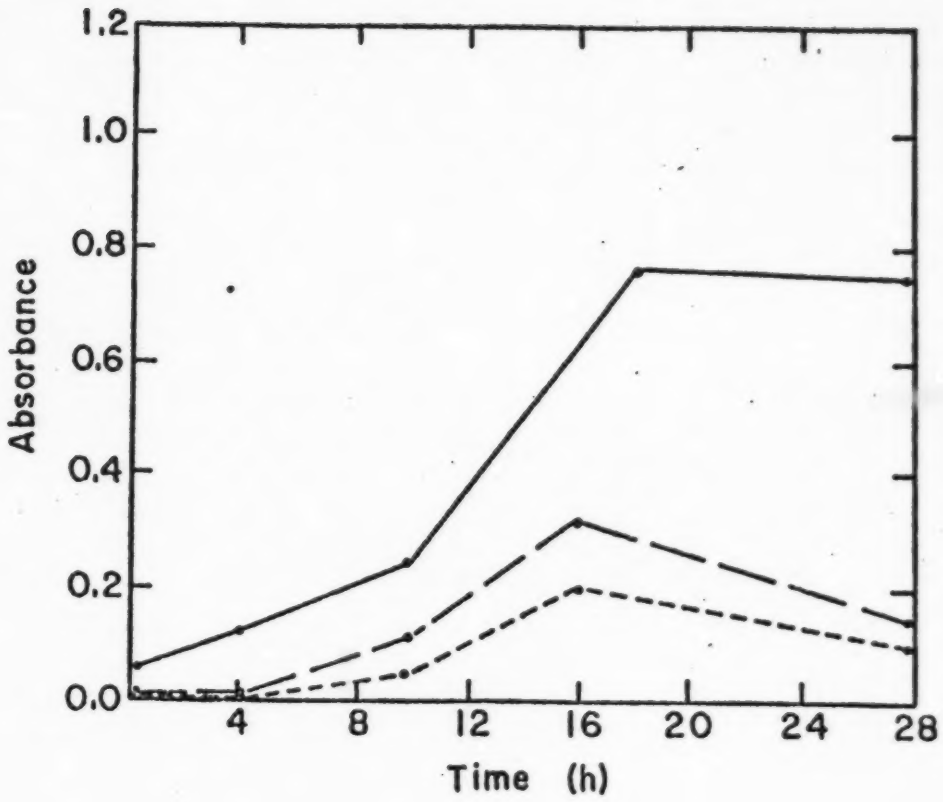


Figure 17. Growth and accumulation of compounds IIA and IIIA by cultures of Beijerinckia B8/36. (legend next page).

monochlorinated dibenzo-p-dioxin dihydrodiols appears to result in the formation of single phenolic products. This phenomenon may be due to resonance stablilization or conformation of the dehydration intermediates and hence may favor the formation of single phenolic products (2,17).

Figure 17 Legend.

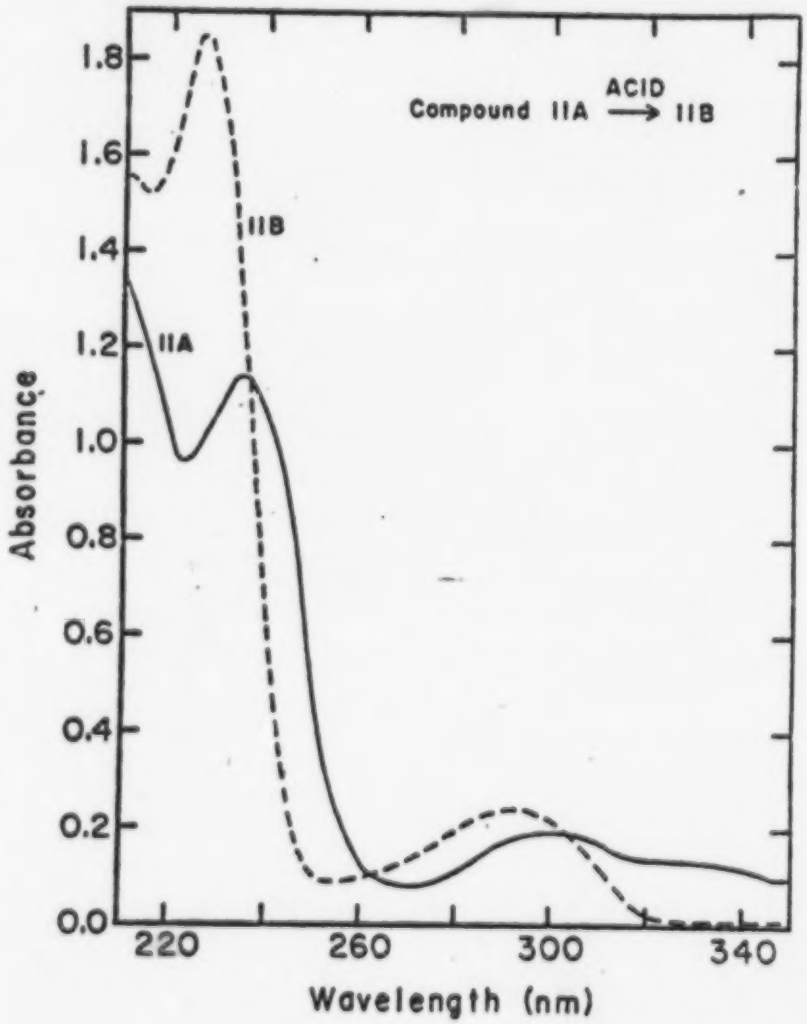
Cultures (500 ml) containing 1-chloro- (II) and 2-chlorodibenzo-p-dioxin (III) were grown as described in the text. Growth of the cultures was monitored turbidometrically at 600 nm (———). Accumulation of compounds IIA and IIIA was measured quantitatively as described in Materials and Methods. Analysis of the ultraviolet spectrum of compound IIA indicated an absorption maximum at 235 nm. Compound IIIA exhibited an absorption maximum at 237 nm. The accumulation of compounds IIA and IIIA with time is expressed as A_{235} (— —) and A_{237} (------) per ml of culture, respectively.

TABLE 8. PHYSICAL PROPERTIES OF THE METABOLITES FORMED FROM MONOCHLORINATED DIBENZO-p-DIOXINS BY BEIJERINCKIA B8/36.

Property	Compound	
	IIA	IIIA
R_f (chloroform:acetone)	0.21	0.21
Gibbs reaction	Brown	Brown
Melting point	130-131 C	n.d.[1]
U.v. maxima (methanol)	235 nm 303 nm	237 nm 309 nm
I.r. maxima (nujol)	3.10 µm 5.95 µm 6.37 µm 9.45 µm	3.07 µm 5.97 µm 6.37 µm 9.45 µm
Mass spectra (m/e)	252 (M^+) 234 ($-H_2O$)	252 (M^+) 234 ($-H_2O$)
Number of Cl	1	1
Proposed structure	$C_{12}H_9O_4Cl$	$C_{12}H_9O_4C$

[1]Not done

Preliminary evidence for a cis-stereochemistry of the hydroxyl groups of the dihydrodiols (compounds IIA and IIIA) was obtained with the syntheses of isopropylidine derivatives. Compounds IIA and IIIA were converted to nonpolar products, compounds IIC (R_f 0.62) and IIIC (R_f 0.62), respectively, when incubated in the presence of 2,2-dimethoxypropane as described above. Both of the latter products absorbed ultraviolet light and gave tan colors with Gibbs re-



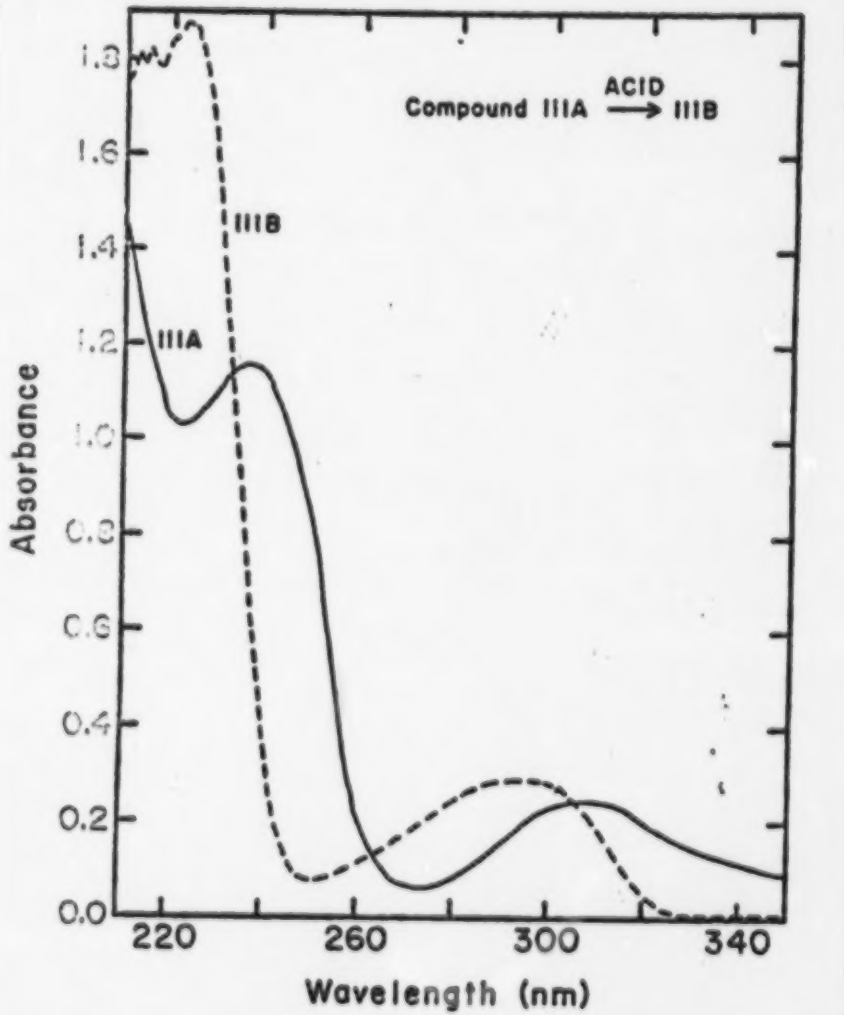


Figure 18. Acid-catalyzed dehydration of compounds IIA and IIIA
(legend next page)

Figure 18 Legend.

Ultraviolet absorption spectra of compounds IIA and IIA was monitored before (solid lines) and after (dashed lines) acidification with H_2SO_4 as described in Materials and Methods. Each compound was isolated and purified by preparative thin-layer chromatography and recrystallized from hexane-acetone. Compounds IIA and IIIA (1.0 mg) were dissolved in 3.0 ml of methanol and the ultraviolet spectrum recorded. Dehydration of compounds IIA and IIIA was initiated with the addition of 10.0 µl of 5.0 N H_2SO_4 and the solutions were incubated at 27 C for 30 min. Reactions were monitored by thin-layer chromatography as described. Upon completion of the acid-catalyzed dehydration the ultraviolet spectrum was again recorded.

agent. The isopropylidene derivatives of compounds IIA and IIIA were found to be highly unstable and decomposed to products resembling compounds IIB and IIIB. These results indicate that cis-dihydrodiols are also the initial metabolites from the oxidation of monochlorinated dibenzo-p-dioxins by Beijerinckia sp.

Oxidation of dibenzo-p-dioxin (I) by Beijerinckia sp., wild type

When the parent strain of Beijerinckia was grown in mineral salts medium containing 0.2% succinate and 0.05% dibenzo-p-dioxin (I), no products were detected by thin-layer chromatography or spectrophotometric analysis of the culture medium. In addition, the growth of the organism was inhibited after 4 hours (Figure 19). To determine if further oxidation was possible, a large-scale culture of Beijerinckia sp. was grown as described with 0.2% succinate and 0.05% biphenyl. The washed cell pellet (5.0 g wet wt) was resuspended in 500 ml of 0.05 M KH_2PO_4 buffer, pH 7.2, containing 0.05% dibenzo-p-dioxin. The resting cell suspension was incubated on a rotary shaker at 27 C. After 12 hours, the suspension was filtered through glass wool to remove unused dibenzo-p-dioxin. The filtrate was adjusted to pH 4.0 with concentrated H_2SO_4 and extracted with two volumes of ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4 and the solvent removed, leaving 21.0 mg of an oily residue. Analysis of the residue by thin-layer chromatography revealed the formation of a neutral product (compound ID; R_f 0.3) that absorbed ultraviolet light and gave an intense brown color with Gibbs reagent. Purification of the residue led to the isolation of 6.0 mg of a crystalline product that melted at 172-173 C. Compound ID was identified as 1,2-dihydroxydibenzo-p-dioxin, having mass, infrared and ultraviolet spectra identical to those of the biological product previously characterized.

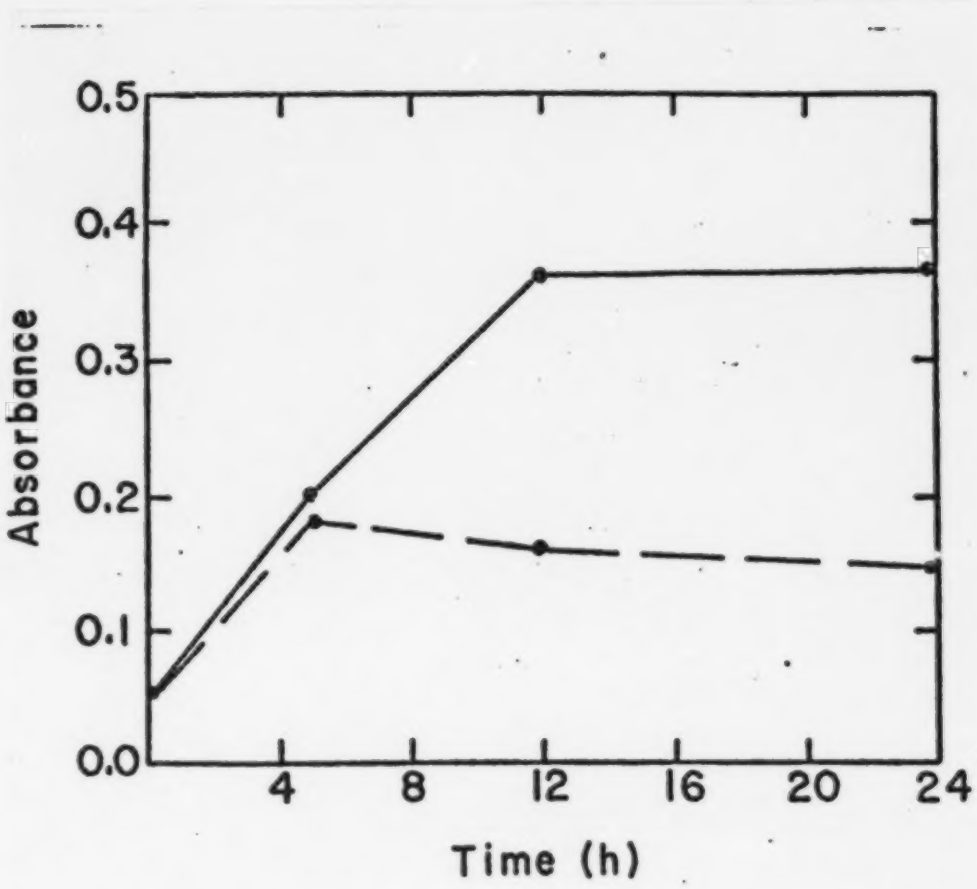


Figure 19. Effect of dibenzo-p-dioxin on the growth of Beijerinckia sp., wild type

It is of interest that growth of the parent strain of Beijerinckia on succinate was inhibited when dibenzo-p-dioxin (0.05%) was included in the culture medium. As cis-1,2-dihydroxy-1,2-dihydrodibenzo-p-dioxin was not inhibitory for the growth of the mutant strain (B8/36) of Beijerinckia, the toxic effects may be due to the oxidation of dibenzo-p-dioxin to 1,2-dihydroxydibenzo-p-dioxin. The toxicity of 1,2-dihydroxydibenzo-p-dioxin was inferred from from the fact that the compound was found to accumulate when resting cell suspensions of the parent strain of Beijerinckia were incubated in the presence

Figure 19 Legend.

Cultures (500 ml) of the parent strain of Beijerinckia were grown in mineral salts broth with 0.2% succinate as the carbon source. Experimental cultures contained 0.05% dibenzo-p-dioxin. Growth of control (————) and experimental (----------) cultures was monitored turbidometrically at 600 nm.

of dibenzo-p-dioxin. Furthermore, growth inhibition of the parent strain was observed after four hours. This period corresponds approximately to the amount of time required by the organism for induction of enzymes necessary for aromatic hydrocarbon oxidation, as indicated by the accumulation of cis-1,2-dihydroxy-1,2-dihydrodibenzo-p-dioxin after four hours by Beijerinckia B8/36.

Oxidation of 1,2-dihydroxydibenzo-p-dioxin by cell extracts

Polarographic experiments with cell extracts were conducted to test the ability of 1,2-dihydroxydibenzo-p-dioxin to serve as a substrate for enzymes responsible for the oxidative fission of the aromatic nucleus (Figure 20).

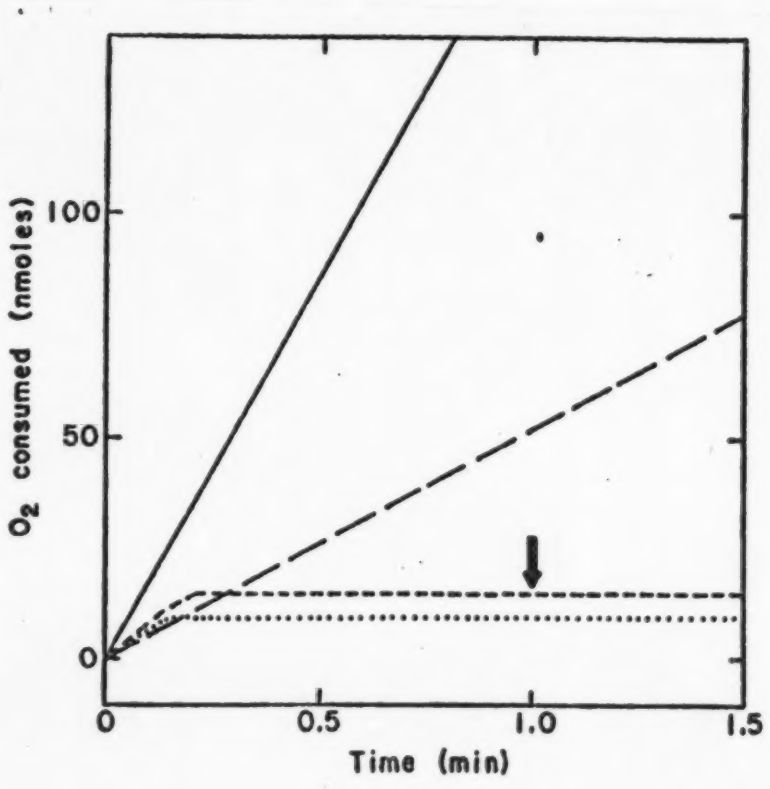


Figure 20. Polarographic assay of ring-fission oxygenases in cell extracts of Beijerinckia sp. (legend next page)

Figure 20 Legend.

The reaction vessel contained, in a total volume of 1.4 ml, 0.05 M KH_2PO_4 buffer, pH 7.5, and cell extract (0.81 mg of protein). The reaction was initiated with the addition of 100 nmoles of catechol (————), 100 nmoles of 2,3-dihydroxybiphenyl (— — —), 100 nmoles of 1,2-dihydroxydibenzo-p-dioxin (--------) and 10 nmoles of 1,2-dihydroxydibenzo-p-dioxin (·····), all in 10 µl of N,N-dimethylformamide. At the time indicated by the arrow (↓), 100 nmoles of catechol or 2,3-dihydroxybiphenyl was added. All rates were corrected for endogenous respiration. No auto-oxidation of the catechol substrates was observed under the assay conditions described.

An initial investigation revealed the cell extracts were active on the physiological substrates, 2,3-dihydroxybiphenyl (64.1 nmoles of O_2 consumed/min per mg of protein) and catechol (212.2 nmoles of O_2 consumed/min per mig of protein). Cell extracts exhibited a brief initial rate of oxidation of 1,2-dihydroxydibenzo-p-dioxin (98.6 nmoles of O_2 consumed/min per mg of protein) and then rapidly fell to the non-enzymatic rate. In addition, incubation of cell extracts (0.81 mg of protein) with as low as 10 nmoles of 1,2-dihydroxydibenzo-p-dioxin led to complete inhibition or inactivation of the catechol and 2,3-dihydroxybiphenyl oxygenases, as additions of 100 nmoles of either substrate did not result in further enzymatic activity.

To determine if both enzymes catalyzing the oxidative fission of the aromatic nucleus were capable of oxidizing 1,2-dihydroxydibenzo-p-dioxin, the 2,3-dihydroxybiphenyl oxygenase was separated from the catechol oxygenase by ion-exchange chromatography (Figure 21). A crude cell extract (0.23 g of protein) was loaded onto a column of DEAE-cellulose (1.4 x 8.0 cm) which had been previously equilibrated with 0.05 M KH_2PO_4 buffer, pH 7.5, containing 10% acetone (phosphate-acetone buffer). The column was washed with 50 ml of phosphate-acetone buffer, and 10 ml fractions were collected. The two activities were eluted separately using a stepwise KCl gradient in phosphate-acetone buffer. 2,3-Dihydroxybiphenyl oxygenase was eluted with 0.2 M KCl in phosphate-acetone buffer (fractions 18-21) and exhibited activity with both 2,3-dihydroxybiphenyl and catechol. The catechol oxygenase was eluted with 0.4 M KCl in phosphate-acetone buffer (fractions 23-28). Fractions from each peak with the highest specific activity were pooled and utilized in further studies.

To determine if the activity present in fractions 18-21 was due to a single protein species, the effect on activity of incubation at 55 C with

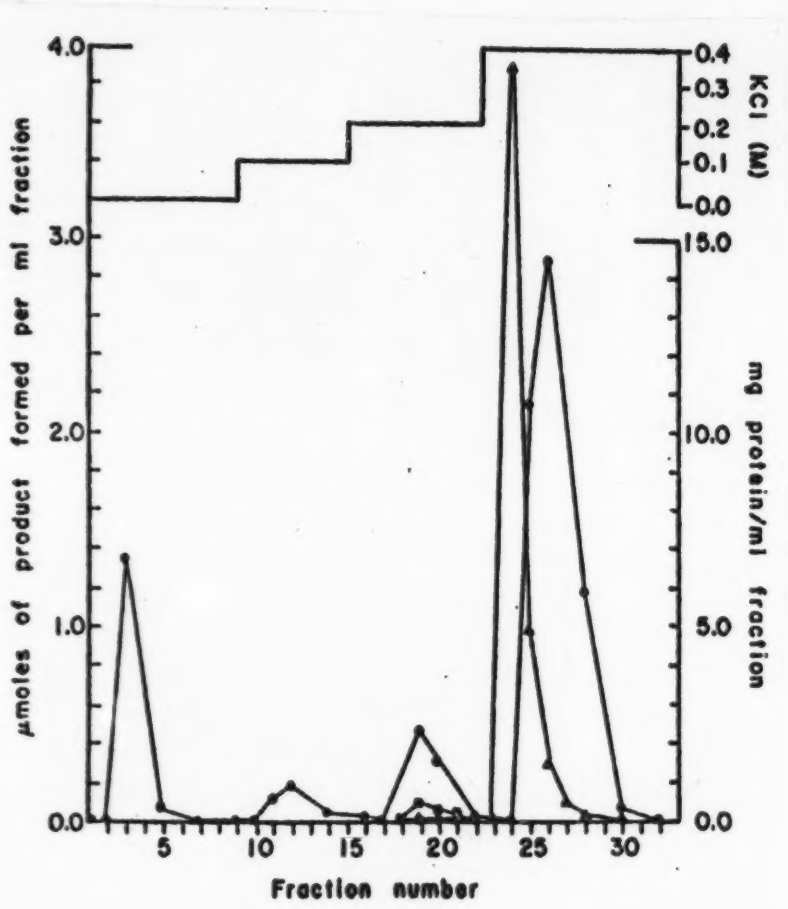


Figure 21. Separation of the 2,3-dihydroxybiphenyl and catechol oxygenases by ion-exchange (DEAE) chromatography (legend next page)

time was investigated. The activity in the fraction for both 2,3-dihydroxybiphenyl and catechol was found to decrease at the same rate with time. Thus, it was assumed that the activities were due to the action of a single enzyme, which was tentatively identified as the 2,3-dihydroxybiphenyl oxygenase.

Polarographic experiments were performed to test the ability of 1,2-dihydroxydibenzo-p-dioxin to serve as a substrate for the partially purified

Figure 21 Legend.

Protein was eluted from the column with a stepwise gradient of 60 ml of 0.1 M KCl in phosphate-acetone buffer, 70 ml of 0.2 M KCl in phosphate-acetone buffer and 120 ml of 0.4 M KCl in phosphate-acetone buffer. Samples (10-100 µl) of each fraction were analyzed spectrophotometrically for oxygenase activity with catechol (▲ - ▲) and 2,3-dihydroxybiphenyl (■ - ■) as described. Enzyme activity is expressed as moles of product formed/min per ml of fraction. Protein concentration (● - ●) in the fractions was determined by the method of Lowry et al. (20).

2,3-dihydroxybiphenyl and catechol oxygenases. The results of the analyses are summarized in Table 9. The 2,3-dihydroxybiphenyl oxygenase (0.48 mg of protein) was capable of oxidizing both 1,2-dihydroxydibenzo-p-dioxin and 2,3-dihydroxybiphenyl, while the catechol oxygenase (0.54 mg of protein) was unable to oxidize 1,2-dihydroxydibenzo-p-dioxin.

Spectrophotometric assays were utilized for kinetic analyses to determine the type of inhibition exhibited by 1,2-dihydroxydibenzo-p-dioxin with respect to the physiological substrates of the partially purified 2,3-dihydroxybiphenyl and catechol oxygenases. All inhibition studies were conducted after pre-incubation of the enzyme with the inhibitor for 1 min prior to initiation of the reaction with the appropriate substrate. Activity of the 2,3-dihydroxybiphenyl oxygenase was monitored using a protein concentration of 0.12 mg/ml. The Michaelis constant (Km) for 2,3-dihydroxybiphenyl was 8.3 μM. The effect of varying concentrations of 2,3-dihydroxybiphenyl was determined in the presence of 1.0 μM 1,2-dihydroxydibenzo-p-dioxin. Results of the analyses were used to prepare a Lineweaver-Burke plot (Figure 22a). Activity of the catechol oxygenase was monitored using a protein concentration of 0.2 mg/ml. The Km for catechol was found to be 7.1 μM. The effect of varying concentrations of catechol was determined in the presence of 0.4 μM 1,2-dihydroxydibenzo-p-dioxin. A Lineweaver-Burke plot was prepared from the results (Figure 22b). These investigations indicate that 1,2-dihydroxydibenzo-p-dioxin is a potent mixed-type inhibitor of both the 2,3-dihydroxybiphenyl and catechol oxygenases present in this organism.

It is noteworthy that the further degradation of 1,2-dihydroxydibenzo-p-dioxin does not appear to be favored due to the inhibition and possibly the inactivation of the ring-fission dioxygenases by this metabolite. A similar

TABLE 9. POLAROGRAPHIC ANALYSIS OF THE ACTIVITIES OF THE PARTIALLY PURIFIED 2,3-DIHYDROXYBIPHENYL AND CATECHOL OXYGENASES.

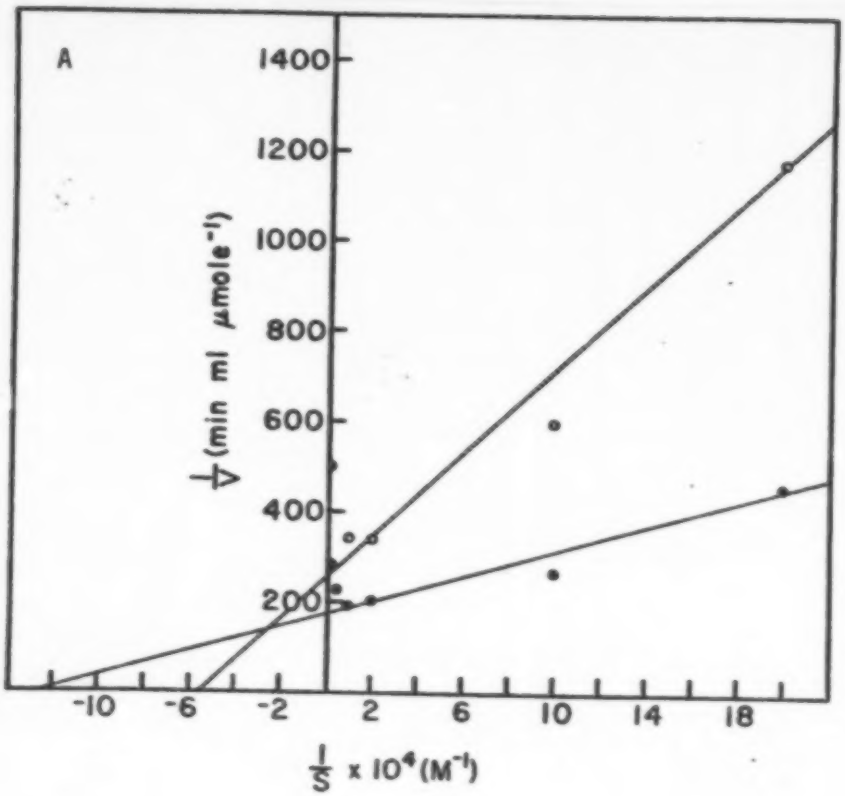
2,3-Dihydroxybiphenyl oxygenase	
Substrate[1]	Specific activity[2]
2,3-Dihydroxybiphenyl	35.6
1,2-Dihydroxydibenzo-p-dioxin	64.4[3]
Catechol oxygenase	
Substrate	Specific activity
Pyrocatechol	206.9
1,2-Dihydroxydibenzo-p-dioxin	0.0

[1]All substrates added in N,N-dimethylformamide (100 nmoles/10.0 µl)

[2]Specific activity (nmoles of O_2 consumed/min per mg of protein)

[3]Initial rate - fell rapidly to non-enzymatic level

phenomenon was observed during the oxidation of dibenzo-p-dioxin by Pseudomonas sp. N.C.I.B. 9816. The present investigation has revealed that 1,2-dihydroxydibenzo-p-dioxin is a potent mixed-type inhibitor of both the 2,3-dihydroxybiphenyl and catechol oxygenases present in the Beijerinckia sp. The nature of this inhibitory phenomenon is under current investigation.



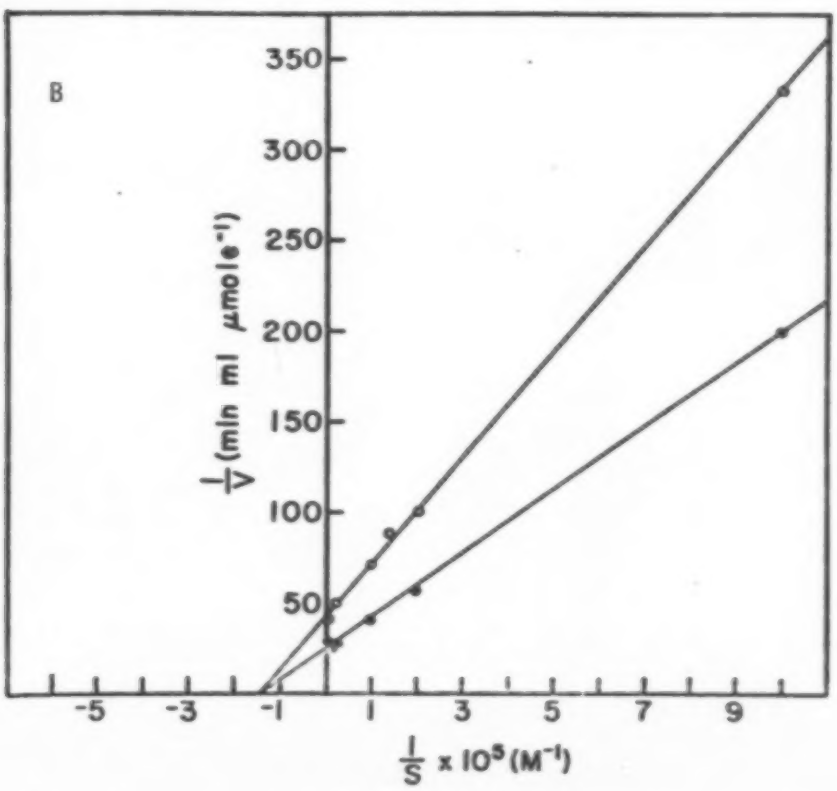


Figure 22. Effect of 1,2-dihydroxydibenzo-p-dioxin on ring-fission oxygenases from Beijerinckia sp. (legends next page)

Figure 21. Effect of 1,2-dihydroxydibenzo-p-dioxin on ring-fission oxygenases from Beijerinckia sp.

A. Effect of 1,2-dihydroxydibenzo-p-dioxin on 2,3-dihydroxybiphenyl oxygenase.

The enzyme was pre-incubated in the presence of 1.0 μM 1,2-dihydroxydibenzo-p-dioxin for 1 min prior to initiation of the reaction with substrate. Activity of the 2,3-dihydroxybiphenyl oxygenase was determined at various concentrations of 2,3-dihydroxybiphenyl in the presence (o —— o) and absence (● —— ●) of the inhibitor.

B. Effect of 1,2-dihydroxydibenzo-p-dioxin on catechol oxygenase.

The enzyme was pre-incubsted in the presence of 0.4 μM 1,2-dihydroxydibenzo-p-dioxin for 1 min prior to initiation of the reaction with substrate. Activity of the catechol oxygenase was determined at various concentrations of catechol in the presence (o —— o) and absence (● —— ●) of the inhibitor.

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